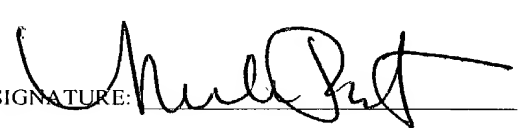


09/03/99 09:30:03  
J017 Rec'd PCT/PTO 30 APR 2001

FORM PTO-1390 (REV 10-94)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER 11613.32USWO
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) Unknown <b>09/830748</b>
INTERNATIONAL APPLICATION NO. PCT/US99/25552	INTERNATIONAL FILING DATE October 29, 1999	PRIORITY DATE CLAIMED October 31, 1998		
TITLE OF INVENTION VARIANTS OF HUMANIZED ANTI-CARCINOMA MONOCLONAL ANTIBODY CC49				
APPLICANT(S) FOR DO/EO/US Syed V.S. KASHMIRI, Eduardo A. PADLAN, Jeffrey SCHLOM				
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:				
<ol style="list-style-type: none"> <li>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</li> <li>3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).</li> <li>4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</li> <li>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) <ol style="list-style-type: none"> <li>a. <input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).</li> <li>b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau.</li> <li>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US)</li> </ol> </li> <li>6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2))</li> <li>7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) <ol style="list-style-type: none"> <li>a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</li> <li>b. <input type="checkbox"/> have been transmitted by the International Bureau.</li> <li>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</li> <li>d. <input checked="" type="checkbox"/> have not been made and will not be made.</li> </ol> </li> <li>8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</li> <li>9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).</li> <li>10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</li> </ol>				
<b>Items 11. to 16. below concern document(s) or information included:</b>				
11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.				
12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.				
13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.				
14. <input type="checkbox"/> A substitute specification.				
15. <input type="checkbox"/> A change of power of attorney and/or address letter.				
16. <input checked="" type="checkbox"/> Other items or information: Form PCT/IPEA/409, Form PCT/ISA/210, Form PCT/IB/304 and 12 pages of Sequence Listing				

U.S. APPLICATION NO (If known, see 37 C.F.R. 1.5) Unknown <b>09/830748</b>		INTERNATIONAL APPLICATION NO PCT/US99/25552		ATTORNEY'S DOCKET NUMBER 11613.32USWO	
17. [X] The following fees are submitted:  <b>BASIC NATIONAL FEE (37 CFR 1.492(a) (1)-(5)):</b> Search Report has been prepared by the EPO or JPO.....\$860.00  International preliminary examination fee paid to USPTO (37 CFR 1.492(a)(1)).....\$690.00  No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) .....\$710.00  Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(3)) paid to USPTO ..... \$1000.00  International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) .....\$100.00				<b>CALCULATIONS</b> <b>PTO USE ONLY</b>	
<b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>				<b>\$860.00</b>	
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				<b>\$0.00</b>	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	47                      -20 =	27	X \$18.00	<b>\$486.00</b>	
Independent claims	5                                -3 =	2	X \$80.00	<b>\$160.00</b>	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$260.00	<b>\$0.00</b>	
<b>TOTAL OF ABOVE CALCULATIONS =</b>				<b>\$1,506.00</b>	
Reduction by 1/2 for filing by small entity, if applicable. Small entity status is claimed pursuant to 37 CFR 1.27				<b>\$</b>	
<b>SUBTOTAL =</b>				<b>\$1,506.00</b>	
Processing fee of <b>\$130.00</b> for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				+ <b>\$0.00</b>	
<b>TOTAL NATIONAL FEE =</b>				<b>\$1,506.00</b>	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40 00 per property				+ <b>\$0.00</b>	
<b>TOTAL FEES ENCLOSED =</b>				<b>\$1,506.00</b>	
				<b>Amount to be: refunded</b>	<b>\$0.00</b>
				<b>charged</b>	<b>\$0.00</b>
a. [X] Check(s) in the amount of <u>\$1,506.00</u> to cover the above fees is enclosed.  b. [ ] Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.  c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>13-2725</u> .					
<b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</b>					
SEND ALL CORRESPONDENCE TO Melissa Jean Pytel MERCHANT & GOULD P.O. Box 2903 Minneapolis, MN 55402-0903			SIGNATURE:   NAME: Melissa Jean Pytel  REGISTRATION NUMBER: 41,512		

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: KASHMIRI et al.  
Docket: 11613.32USWO  
Title: VARIANTS OF HUMANIZED ANTI-CARCINOMA MONOCLONAL ANTIBODY CC49

CERTIFICATE UNDER 37 CFR 1.10

'Express Mail' mailing label number EL669942212US

Date of Deposit: April 30, 2001

I hereby certify that this paper or fee is being deposited with the United States Postal Service 'Express Mail Post Office To Addressee' service under 37 CFR 1.10 and is addressed to the Commissioner for Patents, Washington, D.C. 20231.

By: Yolanda Gray  
Name: Yolanda Gray

BOX PATENT APPLICATION

Commissioner for Patents  
Washington, D.C. 20231

Sir:

We are transmitting herewith the attached:


- ☒ Transmittal sheet, in duplicate, containing Certificate under 37 CFR 1.10.
- ☒ National Stage PCT Patent Application: Spec. 46 pgs; 47 claims; Abstract 1 pgs.  
The fee has been calculated as shown below in the 'Claims as Filed' table.
- ☒ 23 sheets of formal drawings
- ☒ A signed Combined Declaration and Power of Attorney
- ☒ A check in the amount of \$1506.00 to cover the Filing Fee
- ☒ Other: Preliminary Amendment with a marked up copy, Form PCT/IPEA/409, Form PCT/ISA/210, Form PCT/IB/304 and 12 pages of Sequence Listing
- ☒ Return postcard

CLAIMS AS FILED

Number of Claims Filed	In Excess of:	Number Extra	Rate	Fee
<b>Basic Filing Fee</b>				\$860.00
<b>Total Claims</b>				
47	- 20 =	27	x 18.00 =	\$486.00
<b>Independent Claims</b>				
5	- 3 =	2	x 80.00 =	\$160.00
MULTIPLE DEPENDENT CLAIM FEE				\$0.00
TOTAL FILING FEE				\$1506.00

Please charge any additional fees or credit overpayment to Deposit Account No. 13-2725. A duplicate of this sheet is enclosed.

MERCHANT & GOULD P.C.  
P.O. Box 2903, Minneapolis, MN 55402-0903  
(612) 332-5300

By: 

Name: Melissa Jean Pytel  
Reg. No.: 41,512  
Initials: MJP/pjk



(PTO TRANSMITTAL - NEW FILING)

09/830748 09/830748

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

09/830748

Applicant: KASHMIRI et al.

JC18 Rec'd PCT/PTO 3 0 APR 2001

Docket: 11613.32USWO

Title: VARIANTS OF HUMANIZED ANTI-CARCINOMA MONOCLONAL ANTIBODY CC49

## CERTIFICATE UNDER 37 CFR 1.10

'Express Mail' mailing label number EL669942212US

Date of Deposit: April 30, 2001

I hereby certify that this paper or fee is being deposited with the United States Postal Service 'Express Mail Post Office To Addressee' service under 37 CFR 1.10 and is addressed to the Commissioner for Patents, Washington, D.C. 20231

By: Yolanda Gray

Name Yolanda Gray

## BOX PATENT APPLICATION

Commissioner for Patents

Washington, D.C. 20231

Sir:

We are transmitting herewith the attached:

- ☒ Transmittal sheet, in duplicate, containing Certificate under 37 CFR 1.10.
- ☒ National Stage PCT Patent Application: Spec. 46 pgs; 47 claims; Abstract 1 pgs.  
The fee has been calculated as shown below in the 'Claims as Filed' table.
- ☒ 23 sheets of formal drawings
- ☒ A signed Combined Declaration and Power of Attorney
- ☒ A check in the amount of \$1506.00 to cover the Filing Fee
- ☒ Other: Preliminary Amendment with a marked up copy, Form PCT/IPEA/409, Form PCT/ISA/210, Form PCT/IB/304 and 12 pages of Sequence Listing
- ☒ Return postcard

## CLAIMS AS FILED

Number of Claims Filed		In Excess of:		Number Extra		Rate		Fee
Basic Filing Fee								\$860.00
Total Claims								
47	-	20	=	27	x	18.00	=	\$486.00
Independent Claims								
5	-	3	=	2	x	80.00	=	\$160.00
MULTIPLE DEPENDENT CLAIM FEE								\$0.00
TOTAL FILING FEE								\$1506.00

Please charge any additional fees or credit overpayment to Deposit Account No. 13-2725. A duplicate of this sheet is enclosed.

MERCHANT & GOULD P.C.  
P.O. Box 2903, Minneapolis, MN 55402-0903  
(612) 332-5300

By: Melissa Jean Pytel  
Name: Melissa Jean Pytel  
Reg. No.: 41,512  
Initials: MJP/pjk



(PTO TRANSMITTAL - NEW FILING)



**KLARQUIST SPARKMAN, LLP**

16th Floor World Trade Center, 121 S.W. Salmon Street, Portland, Oregon 97204 U.S.A.

PHONE: 503-226-7391 FAX: 503-228-9446

**PLEASE DELIVER DIRECTLY TO EXAMINER****Fax No.:** (703) 305-3230**Total No. Pages:** 10 including this cover sheet

**Message:** Transmitted herewith for filing in the above-identified application is a Revocation of Prior Power of Attorney and New Power of Attorney by Assignee and Certificate by Assignee Under 37 C.F.R. 3.73. If you do not receive all pages or if you have problems receiving transmittal, please call William D. Noonan, M.D., at (503) 226-7391. The fee (large entity) has been calculated as shown below.

In re application of: Kashmiri et al.

Application No.: 09/830,748

Filed: April 30, 2001

For: VARIANTS OF HUMANIZED ANTI-CARCINOMA MONOCLONAL ANTIBODY CC49

Examiner: Not yet assigned

Art Unit: Not yet assigned

Date: January 8, 2002

CERTIFICATE OF FACSIMILE TRANSMISSION

I hereby certify that this correspondence and any documents referred to as being transmitted herewith are being facsimile transmitted to attention of BPX PCT, U.S. Patent and Trademark Office, at the facsimile telephone number (703) 305-3230 on January 8, 2002.



William D. Noonan, M.D.  
Attorney for Applicant

FEE CALCULATION FOR CLAIMS AS AMENDED

For	No. after amendment	No. paid for previously	Extra	Rate	Fee
Total Claims		- * =		\$18.00	\$ 0.00
Indep.		- ** =		\$84.00	\$ 0.00

<<One/Two/Three>>-month Extension of Time (1 month = \$110.00 2 months = \$400.00 3 months = 920.00)

**TOTAL FEE FOR THIS AMENDMENT** **\$0.00**

\*greater of twenty or number for which fee has been paid. \*\*greater of three of number for which fee has been paid.

- ☒ No additional fee is required.
- ☒ Please charge this fee and any additional fees that may be required in connection with filing of this Revocation of Prior Power of Attorney and New Power of Attorney by Assignee and Certificate by Assignee Under 37 C.F.R. 3.73 to Deposit Account 02-4550.



William D. Noonan, M.D.

Registration No. 30,878

January 8, 2002

Date

cc: Docketing

09/830748  
JC18 Rec'd PCT/PTO 3 0 APR 2001

S/N unknown

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Kashmiri et al.	Docket No.:	11613.32USWO
Serial No.:	unknown	Filed:	concurrent herewith
Int'l Appln No.:	PCT/US99/25552	Int'l Filing Date:	October 29, 1999
Title:	VARIANTS OF HUMANIZED ANTI-CARCINOMA MONOCLONAL ANTIBODY CC49		

PRELIMINARY AMENDMENT

Box PCT  
Assistant Commissioner for Patents  
Washington, D. C. 20231

Dear Sir:

In connection with the above-identified application filed herewith, please enter the following preliminary amendments, a copy of which is enclosed herewith:

IN THE ABSTRACT

Insert the attached Abstract page into the application as the last page thereof.

IN THE SPECIFICATION

A courtesy copy of the present specification is enclosed herewith. However, the World Intellectual Property Office (WIPO) copy should be relied upon if it is already in the U.S. Patent Office.

IN THE CLAIMS

36. (AMENDED) A nucleic acid sequence expressing the humanized antibody of claim 1.
37. (AMENDED) A vector expressing the humanized antibody of claim 1.

$$\begin{array}{ccccccc} \text{H} & \text{H} & \text{H} & \text{H} & \text{H} & \text{H} & \text{H} \\ | & | & | & | & | & | & | \\ \text{C}_1 & -\text{C}_2 & -\text{C}_3 & -\text{C}_4 & -\text{C}_5 & -\text{C}_6 & -\text{C}_7 \\ | & | & | & | & | & | & | \\ \text{H} & \text{H} & \text{H} & \text{H} & \text{H} & \text{H} & \text{H} \end{array}$$

38. (AMENDED) A composition for treatment of cancer, comprising the humanized antibody of claim 1.

39. (AMENDED) A composition for detecting cancer cells, comprising the humanized antibody of claim 1.

40. (AMENDED) A composition of for detecting cancer cells, comprising a polypeptide capable of specifically binding TAG-72, said polypeptide comprising a functional fragment of the humanized antibody of claim 1.

42. (AMENDED) A method for treating cancer comprising:  
administering the humanized antibody of claim 1 to a patient.

43. (AMENDED) A method of detecting cancer cells, comprising:  
contacting cells with the humanized antibody of claim 1.

46. (AMENDED) A method of detecting cancer cells, comprising:  
contacting cells with composition comprising a polypeptide  
capable of specifically binding TAG-72, said polypeptide comprising a functional  
fragment of the humanized antibody of claim 1.

## REMARKS

The above preliminary amendment is made to remove multiple dependencies from claims 36-40, 42, 43 and 46.

A new abstract page is supplied to conform to that appearing on the publication page of the WIPO application, but the new Abstract is typed on a separate page as required by U.S. practice.

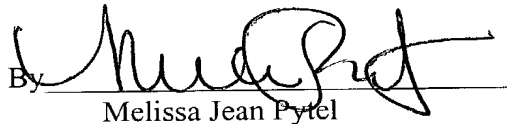
Applicants respectfully request that the preliminary amendment described herein be entered into the record prior to calculation of the filing fee and prior to examination and consideration of the above-identified application.

If a telephone conference would be helpful in resolving any issues concerning this communication, please contact Applicants' primary attorney-of record, Melissa Jean Pytel (Reg. No. 41,512), at (612) 371.5304.

Respectfully submitted,

MERCHANT & GOULD P.C.  
P.O. Box 2903  
Minneapolis, Minnesota 55402-0903  
(612) 332-5300

Dated: April 30, 2001

By   
Melissa Jean Pytel  
Reg. No. 41,512

MJP/pjk

MARKED UP COPY

36. A nucleic acid sequence expressing the humanized antibody of [any of claims 1, 11, 23, 34 or 35] claim 1.
37. A vector expressing the humanized antibody of [any of claims 1, 11, 23, 34 or 35] claim 1.
38. A composition for treatment of cancer, comprising the humanized antibody of [any of claims 11, 11, 23, 34 or 35] claim 1.
39. A composition for detecting cancer cells, comprising the humanized antibody of [claims 1, 11, 23, 34 or 35] claim 1.
40. A composition of for detecting cancer cells, comprising a polypeptide capable of specifically binding TAG-72, said polypeptide comprising a functional fragment of the humanized antibody of [any of claims 1, 11, 23, 34 or 35] claim 1.
42. A method for treating cancer comprising:  
administering the humanized antibody of [any of claims 1, 11, 23, 34 or 35] claim 1 to a patient.
43. A method of detecting cancer cells, comprising:  
contacting cells with the humanized antibody of [any of claims 1, 11, 23, 34 or 35] claim 1.
46. A method of detecting cancer cells, comprising:  
contacting cells with composition comprising a polypeptide capable of specifically binding TAG-72, said polypeptide comprising a functional fragment of the humanized antibody of [any of claims 11, 11, 23, 34 or 35] claim 1.

### **ABSTRACT**

The invention is directed towards mouse-human chimeric variants of CC49 monoclonal antibodies with minimal murine content. A first aspect of the invention provides CDR variants of humanized monoclonal antibody (HuCC49) in which less than all six (three heavy chain and three light chain) Complementarity Determining Regions (CDRs) of CC49 are present. A second aspect of the invention provides SDR variants of humanized monoclonal antibody (HuCC49) in which only Specificity Determining Regions (SDRs) of at least one CDR from CC49 are present. The invention is also directed towards biotechnological methods of making the variants and therapeutic methods of using the variants.

**VARIANTS OF HUMANIZED ANTI-CARCINOMA  
MONOCLONAL ANTIBODY CC49****Background**

5       Antibodies are specific immunoglobulin (Ig) polypeptides produced by the vertebrate immune system in response to challenges by foreign proteins, glycoproteins, cells, or other antigenic foreign substances. The binding specificity of such polypeptides to a particular antigen is highly refined, with each antibody being almost exclusively directed to the particular antigen which elicited it. This  
10       specificity resides in the structural complementarity between the antibody combining site and the antigenic determinant. Antibody combining sites are made up of residues that are primarily from the hypervariable or complementarity determining regions (CDRs). Occasionally, residues from nonhypervariable or framework regions do influence the overall domain structure and hence the combining site.

15       There are two major methods for generating vertebrate antibodies: generation of polyclonal antibodies *in situ* by mammalian B lymphocytes and generation of monoclonal antibodies in cell culture by B cell hybrids.

      To generate antibodies *in situ*, an animal (such as a mouse or rabbit) is injected with an antigen. Several weeks later, blood is drawn from the animal and  
20       centrifuged. The resulting serum contains antibodies against the injected antigen. The resulting antibodies are polyclonal antibodies because they are products of many different populations of antibody producing cells and hence differ somewhat in their precise specificity and affinity for the antigen.

      Monoclonal antibodies are produced using hybridoma technology in which  
25       an antibody producing cell is fused with a tumor cell that has the capacity for unlimited proliferation. In contrast to polyclonal antibodies, monoclonal antibodies are homogeneous because they are synthesized by a population of identical cells that are derived from a single hybridoma cell.

      However, the use of monoclonal antibodies in humans is severely restricted  
30       when the monoclonal antibody is produced in a non-human animal. Repeated injections in humans of a "foreign" antibody, such as a mouse antibody, may lead to harmful hypersensitivity reactions, i.e., anti-mouse antibody (HAMA) or an anti-idiotypic response. The HAMA response makes repeated administrations ineffective due to an increased rate of clearance from the patient's serum and/or  
35       allergic reactions by the patient.

Attempts have been made to manufacture human-derived monoclonal antibodies using human hybridomas. Unfortunately, yields of monoclonal antibodies from human hybridoma cell lines are relatively low compared to mouse hybridomas. Additionally, human cell lines expressing immunoglobulins are relatively unstable compared to mouse cell lines, and the antibody producing capability of these human cell lines is transient. Thus, while human immunoglobulins are highly desirable, human hybridoma techniques have not yet reached the stage where human monoclonal antibodies with the required antigenic specificities can be easily obtained.

Thus, antibodies of non-human origin have been genetically engineered to create chimeric or humanized antibodies. Such genetic engineering results in antibodies with a reduced risk of a HAMA response compared to that expected after injection of a human patient with a mouse antibody. For example, chimeric antibodies can be formed by grafting non-human variable regions to human constant regions. Khazaeli et al. (1991), J. Immunotherapy 15:42-52. Generally humanized antibodies, are formed by grafting non-human complementarity determining regions (CDRs) onto human framework regions (FRs) (See European Patent Application 0 239 400; Jones et al. (1986), Nature (London), 321:522-525; and Reichman et al. (1988), Nature (London), 332:323-327). Typically, humanized monoclonal antibodies are formed by grafting all six (three light chain and three heavy chain) CDRs from a non-human antibody into Framework Regions (FRs) of a human antibody. Alternately, Fv antibodies (See United States Patent 4,642,334) or single chain Fv (SCFV) antibodies (See United States Patent 4,946,778) can be employed to reduce the risk of a HAMA response.

However, these modified antibodies still retain various non-human light and heavy chain variable regions: the chimeric, Fv and single chain Fv antibodies retain entire non-human variable regions and CDR-grafted antibodies retain CDR of non-human origin. Such non-human regions can elicit an immunogenic reaction when administered to a human patient. Thus, many humanized MAbs remain immunogenic in both subhuman primates and in humans, with the humoral response of the host directed towards the variable region of these MAb (Hakimi et al. (1991), J. Immunol., 147:1352-1359; Stephens et al. (1995), Immunology, 85:668-674; Singer et al. (1993), J. Immunol., 150:2844-2857; and Sharkey et al. (1995), Cancer Res. 55:5935s-5945s).



One known human carcinoma tumor antigen is tumor associated glycoprotein-72 (TAG-72), as defined by monoclonal antibody B72.3 (See Thor et al., (1986) Cancer Res., 46:3118-3124; and Johnson et al., (1986), Cancer Res., 46:850-85). TAG-72 is associated with the surface of certain tumor cells of human origin.

Numerous murine monoclonal antibodies have been developed which have binding specificity for TAG-72. Exemplary murine monoclonal antibodies include the "CC" (colon cancer) monoclonal antibodies, which are a library of murine monoclonal antibodies developed using TAG-72. Certain CC antibodies have been deposited with the ATCC, including CC49 (ATCC No. HB 9459). Monoclonal antibody (MAb) CC49 is a second-generation antibody of B72.3 that reacts with the pancarcinoma tumor-associated antigen, TAG-72. Radiolabeled MAb CC49 has been shown to target tumor in both animal models and in ongoing radioimmunotherapeutic and radioimmunodiagnostic clinical trials. (Divgi et al. (1994) Nucl. Med. Biol., 21:9-15; Meredith et al. (1994), J. Nucl. Med., 35:1017-1022; Mulligan et al. (1995), Clin. Cancer Res., 1:1447-1454; Arnold et al. (1992), Ann. Surgery, 216:627-632) The potential clinical utility of MAb CC49 is evident both from animal studies and ongoing clinical trials with the antibody. However, patients administered MAb CC49 do generate HAMA responses (Divgi et al., (1994) Nuc. Med. Biol., 21:9-15); Mulligan et al., (1995) Clin. Cancer Res., 1:1447-1454).

A humanized monoclonal antibody (HuCC49) has been formed by grafting hypervariable regions from monoclonal antibody CC49 into variable light (V<sub>L</sub>) and variable heavy (V<sub>H</sub>) frameworks of human monoclonal antibodies LEN and 21/28' CL, respectively, while retaining murine framework residues required for integrity of the antigen combining-site structure. (See, Kashmiri et al., (1995) Hybridoma, 14(5):461-473). This HuCC49 was shown to bind the TAG-72 antigen, albeit with a lower affinity, and demonstrated equivalent tumor targeting in animal models bearing human tumor xenografts.

It has been shown that not all residues of CDRs are critical in the complementarity of antigen/antibody surfaces. Known structures of the antigen-antibody complexes suggests that only 20-33% of CDR residues are involved in antigen contact (Padlan, (1994) Mol. Immunol., 31:169-217). A comprehensive analysis of the available data of the sequences and the three dimensional structure of antibody combining sites has helped identify CDR residues that may be most critical in the antigen antibody interaction (Padlan et al., (1995) FASEB J., 9:133-139).



**Brief Description of the Figures**

Figure 1 illustrates a basic immunoglobulin structure.

Figure 2 shows a comparison of the CDR sequences of murine MAb CC49 and human MAbs LEN and 21/28'CL. Amino acid residues are numbered using the convention of Kabat et al. The underlined numbers indicate the specificity determining residues (SDRs).

Figure 3 is a schematic representation of the eukaryotic expression constructs of the humanized heavy (B) chains of HuCC49. Thin lines represent sequences derived from the prokaryotic vectors pBR322, pBluescript SK<sup>+</sup>, or pCR II. Thick lines depict human  $\gamma$  constant region. Boxes with vertical, horizontal, or cross bars show neomycin, mycophenolic acid, or hygromycin resistance genes; thin arrows show their transcriptional direction. Empty boxes are retroviral long terminal repeats, while thick arrows show the HCMV promoter and its transcriptional direction. Only relevant enzyme sites are shown. A: *Apal*; B: *Bam*HI; C: *Clal*; Hd: *Hind*III; Hp: *Hpa*I; N: *Nhe*I; R: *Eco*RI; and S: *Sac*II.

Figure 4 is a schematic representation of the eukaryotic expression constructs of the humanized light chains of HuCC49. As with Figure 3, thin lines represent sequences derived from the prokaryotic vectors pBR322, pBluescript SK<sup>+</sup>, or pCR II. Thick lines depict human  $\kappa$  constant region. Boxes with vertical, horizontal, or cross bars show neomycin, mycophenolic acid, or hygromycin resistance genes; thin arrows show their transcriptional direction. Empty boxes are retroviral long terminal repeats, while thick arrows show the HCMV promoter and its transcriptional direction. Only relevant enzyme sites are shown. A: *Apal*; B: *Bam*HI; C: *Clal*; Hd: *Hind*III; Hp: *Hpa*I; N: *Nhe*I; R: *Eco*RI; and S: *Sac*II.

Figure 5 is a schematic representation of the dual expression constructs of the variant heavy (H) and light (L) chain genes derived from the baculovirus vector pAcUW51. P10 and *polh* represent p10 and polyhedrin promoter; arrows show their direction of transcription. Ori and fl are SV40 and fl origin of replication. Amp<sup>R</sup> represents an ampicillin resistant gene.

Figure 6 shows an SDS-PAGE analysis of purified MAb HuCC49 and its variants. All samples are shown in a reduced condition. Lane 1: molecular weight marker (Gibco Brl); Lanes 2-8: variants L-1, L-2, L-3, L-1,2, H-1, H-2 and H-3; Lane 9: HuCC49.

Figure 7 shows an analysis of parental and variant forms of HuCC49 in a competitive RIA. The antigen binding of the light chain (A) and heavy chain (B) CDR variants was assessed using  $^{125}\text{I}$ -labeled HuCC49. In panel A, the competitors were: HuCC49, L-1, L-2, L-3, L-1,2. In panel B, the competitors were: H-1, H-2 and H-3.

Figure 8 shows the effect of light chain CDRs on binding of anti-idiotypic MAbs. The HuCC49 CDR variants were characterized in a competition RIA with  $^{125}\text{I}$ -HuCC49 and CC49 anti-idiotypic MAbs AI49-3 (panel A), AI49-1 (panel B) and AI49-8 (panel C). The competitors were: HuCC49, L-1, L-2, L-3, L-1,2.

Figure 9 shows the effect of heavy chain CDRs on binding of anti-idiotypic MAbs. The HuCC49 CDR variants were characterized in a competition RIA with  $^{125}\text{I}$ -CC49 and CC49 anti-idiotypic MAbs AI49-3 (panel A), AI49-1 (panel B) and AI49-8 (panel C). The competitors were: HuCC49, H-1, H-2, H-3.

Figure 10 shows an analysis of human anti-idiotypic antibodies to HuCC49 variants using a competitive RIA by HPLC methodology. A patient's anti-idiotypic response to CC49 was characterized using purified parental HuCC49 and CDR variants as competitors with  $^{125}\text{I}$ -HuCC49. The inability of a variant to inhibit complex formation of the patient's sera with the  $^{125}\text{I}$ -HuCC49 indicates that the CDR replaced from the variant was immunogenic to the patient. In panel A, the competitors were: HuCC49, L-1, L-2, L-3, L-1,2. In panel B, the competitors were: H-1, H-2 and H-3.

Figure 11 shows the amino acid sequences of  $V_L$  frameworks of human MAb LEN and humanized  $V_L$  of CC49 (HuCC49) in panel A. Panel B shows the amino acid sequences of  $V_H$  frameworks of human MAb 21/28'CL and humanized  $V_H$  of CC49 (HuCC49). Framework residues that are deemed to be important in maintaining the combining site structure of CC49 are marked by an asterisk.

Figure 12 shows the nucleotide sequence of HuCC49 variable light ( $V_L$ ) and variable heavy ( $V_H$ ) region genes in panels A and B, respectively. Sequences of flanking oligomers that do not encode the variable region domains or their leader peptides are shown in lowercase letters. The  $V_L$  region (A) is encoded by nucleotides from positions 74 to 412, while nucleotides from position 70 to 415 (B) comprise the  $V_H$  region.

Figure 13 is a graph of the results of a competition assay using variants of HuCC49.

Figure 14 shows the results of an HPLC analysis of patient reactivity to variants of HuCC49. Competitors were at 5 $\mu$ g per reaction. The values are the percent of complexes, the higher molecular weight species, resolved by size-exclusion chromatography. Complex formation indicates removal of the epitope recognized by the patient's antibody. Inhibition of complex formation indicates that the immunogenic epitope is still present in the HuCC49 variant.

Figure 15 is a graph showing the comparison of patient reactivity with HuCC49 and various variants thereof.

Figure 16 is graph showing the immunoreactivity of variant  $^{97}\text{L}_{1,2}/^{60-62,64}\text{H}$ .

Figure 17 is a graph of the pharmacokinetics of plasma retention of HuCC49 and a variant thereof.

Figure 18 is a table showing the biodistribution of i.v. administered radiolabeled HuCC49 and variants in athymic mice bearing LS-174T human colon carcinoma xenografts. Athymic mice bearing LS-174T human colon carcinoma xenografts (s.c.) were coinjected with 1.4 $\mu$ Ci of  $^{131}\text{I}$ -HuCC49 and 4.4 of  $^{125}\text{I}$ -Variant. The mice were sacrificed at the timepoints indicated, the organs harvested, wet-weighted and the radioactivity detected in a  $\gamma$ -scintillation counter. The percent weight injected dose per gram for each tissue was calculated. The standard error of the mean was also calculated and were 0.06%ID/g or less.

Figure 19. HPLC analysis of patient HAMA following intravenous injection of  $^{177}\text{Lu}$ -CC49.

Figure 20. HPLC analysis of patients' humoral response to the variable region of MAb CC49. The percent complex formation has been plotted versus time for (solid lines) patients DS (O), LW ( $\square$ ), JJ ( $\Delta$ ), DG ( $\bullet$ ), LJ ( $\blacksquare$ ), TD ( $\blacktriangle$ ); (dotted lines) JG (O), RW ( $\square$ ), JM ( $\Delta$ ), EA ( $\bullet$ ), CP ( $\blacksquare$ ), LQ ( $\blacktriangle$ );

Figure 21. Detection of patient anti-idiotypic antibody response to murine CC49.

Figure 22. HPLC analysis demonstrating CDR specificity of patient LQ.

### **Definitions**

Prior to setting forth the invention, definitions of certain terms which are used in this disclosure are set forth below:

**Antibody:** This refers to single chain, two-chain, and multi-chain proteins and glycoproteins belonging to the classes of polyclonal, monoclonal, chimeric and hetero immunoglobulins (monoclonal antibodies being preferred); it also includes

synthetic and genetically engineered variants of these immunoglobulins. "Antibody fragment" includes Fab, Fab', F(ab')<sub>2</sub>, and Fv fragments, as well as any portion of an antibody having specificity toward a desired target epitope or epitopes.

Chimeric antibody: This refers to an antibody which includes sequences  
5 derived from two different antibodies, which typically are of different species. Most typically, chimeric antibodies include human and murine antibody fragments, generally human constant and murine variable regions.

Humanized antibody: This refers to an antibody derived from a non-human  
10 antibody, typically murine, and a human antibody which retains or substantially retains the antigen-binding properties of the parent antibody but which is less immunogenic in humans.

Complementarity Determining Region, or CDR: This refers to amino acid  
sequences which together define the binding affinity and specificity of the natural Fv  
region of a native immunoglobulin binding site. The light and heavy chains of an  
15 immunoglobulin each have three CDRs, designated L-CDR1, L-CDR2, L-CDR3  
and H-CDR1, H-CDR2, H-CDR3, respectively. By definition, the CDRs of the light chain are bounded by the residues at positions 24 and 34 (L-CDR1), 50 and 56 (L-CDR2), 89 and 97 (L-CDR3); the CDRs of the heavy chain are bounded by the residues at positions 31 and 35b (H-CDR1), 50 and 65 (H-CDR2), 95 and 102 (H-  
20 CDR3), using the numbering convention delineated by Kabat et al., (1991)

Sequences of Proteins of Immunological Interest, 5th Edition, Department of Health and Human Services, Public Health Service, National Institutes of Health, Bethesda (NIH Publication No. 91-3242).

Framework Region: This refers to amino acid sequences interposed between  
25 CDRs. These portions of the antibody serve to hold the CDRs in an appropriate orientation for antigen binding.

Specificity Determining Residue, or SDR: This refers to amino acid residues of an immunoglobulin that are directly involved in antigen contact.

Constant Region: This refers to the portion of the antibody molecule which  
30 confers effector functions. In the present invention, the variant antibodies include constant regions derived from human immunoglobulins. The heavy chain constant region can be selected from any of five isotypes: alpha, delta, epsilon, gamma or mu. Heavy chains of various subclasses (such as the IgG subclass of heavy chains) are responsible for different effector functions. Thus, by choosing the desired heavy  
35 chain constant region, humanized antibodies with the desired effector function can

be produced. The light chain constant region can be of the kappa or lambda type, preferably the kappa type.

Mammals: This refers to animals that nourish their young with milk secreted by mammary glands, preferably warm blooded mammals.

5        Immunogenicity: A measure of the ability of a targeting protein or therapeutic moiety to elicit an immune response (humoral or cellular) when administered to a recipient. The present invention is concerned with the immunogenicity of humanized antibody CC49.

10       Reduced immunogenicity: This refers to an antibody, typically humanized, that exhibits reduced immunogenicity relative to the parent antibody.

Immunoreactivity: A measure of the ability of an immunoglobulin to recognize and bind to a specific antigen.

15       Substantially similar binding properties: This refers to a humanized antibody which retains the ability to specifically bind the antigen recognized by the parent antibody used to produce the humanized antibody. Preferably, the affinity of the humanized antibody is at least about 10% of the affinity of the parent antibody, more preferably at least about 25%, even more preferably at least about 50%. Most preferably, the humanized antibody exhibits antigen-binding affinity that is at least about 75% of the affinity of the parent antibody. Methods for assaying antigen-binding affinity are well known in the art and include half-maximal binding assays, competition assays, and Scatchard analysis.

20       Substantially Homologous: Refers to immunoglobulin sequences that exhibit at least about 85% identity, more preferably about 90% identity, most preferably about 95% identity with a reference immunoglobulin, wherein % identity is determined by comparing the number identical of amino acid residues between the two immunoglobulins, wherein the positions of the amino acid residues are indicated using the Kabat numbering scheme.

25       Nomenclature: Nucleic acids, amino acids, peptides, protective groups, active groups and so on, when abbreviated, are abbreviated according to the IUPAC IUB (Commission on Biological Nomenclature) or the practice in the fields concerned.

### Detailed Description

30       To facilitate understanding of the invention, a discussion of the structure of a typical antibody molecule will first be provided. The basic immunological structural

unit is shown in Figure 1. Antibodies (also referred to as immunoglobulins) are constructed from four polypeptide chains, two heavy chains and two light chains. The two heavy chains are linked to each other by disulfide bonds and each heavy chain is linked to a light chain by a disulfide bond. There are two types of light chain, lambda ( $\lambda$ ) and kappa ( $\kappa$ ). There are five main heavy chain classes (or isotypes) which determine the functional activity of an antibody molecule: IgM, IgD, IgG, IgA and IgE.

Each chain contains distinct sequence domains. The light chain includes two domains, a variable domain ( $V_L$ ) and a constant domain ( $C_L$ ). The heavy chain includes four domains, a variable domain ( $V_H$ ) and three constant domains ( $C_{H1}$ ,  $C_{H2}$  and  $C_{H3}$ , collectively referred to as  $C_H$ ). The variable regions of both light ( $V_L$ ) and heavy ( $V_H$ ) chains determine binding recognition and specificity to the antigen. The constant region domains of the light ( $C_L$ ) and heavy ( $C_H$ ) chains confer important biological properties such as antibody chain association, secretion, transplacental mobility, complement binding, and binding to Fc receptors. The Fv fragment is the N-terminal part of the Fab fragment of an immunoglobulin consisting of the variable portions of one light chain and one heavy chain. The specificity of the antibody resides in the structural complementarity between the antibody combining site and the antigenic determinant. Antibody combining sites are made up of residues that are primarily from the hypervariable or complementarity determining regions (CDRs). Occasionally, residues from nonhypervariable or framework regions (FR) influence the overall domain structure and hence the combining site.

The variants of the invention are derived from a humanized CC49 (referred to as "parental HuCC49"). Parental HuCC49 is formed by grafting all six (three heavy chain and three light chain) MAb CC49 hypervariable regions onto the variable light ( $V_L$ ) and variable heavy ( $V_H$ ) frameworks of the human MAbs LEN and 21/28<sup>CL</sup>, respectively, while retaining murine framework residues that may be required for the integrity of the antigen combining site structure (Figure 11). (Kashmiri et al., (1995) Hybridoma, 14:461-473). The variants of the invention contain a reduced murine content, and consequently, reduced immunogenicity, when compared to HuCC49. Nonetheless, the variants of the invention retain a binding affinity that is substantially similar to that of HuCC49. Preferably the binding affinity is at least about  $10^8 M^{-1}$ . As used herein, HuCC49 refers to the humanized



antibody formed by Kashmiri et al. The terms "variant HuCC49" or "variant" refer to the immunoglobulins of the invention.

5 A first aspect of the invention provides CDR variants of humanized monoclonal antibody (HuCC49) in which less than all six (three heavy chain and three light chain) Complementarity Determining Regions (CDRs) of CC49 are present. A second aspect of the invention provides SDR variants of humanized monoclonal antibody (HuCC49) in which only Specificity Determining Regions (SDRs) of at least one CDR from CC49 are present.

10 CDR Variants

According to the invention, CDR variants are formed by replacing at least one CDR of CC49 in HuCC49 with a corresponding CDR from a human antibody. Preferably, the L-CDR1 or L-CDR2, or both, from CC49 are replaced by a corresponding CDR from a human antibody. The inventors have found that a variant  
15 in which any of L-CDR3, H-CDR1, H-CDR2 or H-CDR3 of CC49 are replaced by a corresponding CDR from a human antibody do not retain significant binding affinity.

Binding Affinity of CDR Variants

20 According to the invention, CDR variants in which L-CDR1 or L-CDR2 of CC49, or both, are replaced by a corresponding CDR from a human antibody retain biological activity that is substantially similar to the binding affinity of the parental CC49. Generally, the CDR variants of the invention have a binding affinity that is about 25% to about 50% if the binding affinity of the parental CC49, more  
25 preferably about 50% to about 75%, most preferably, about 75% to about 100%.

CDR variants in which H-CDR2 is replaced by a corresponding CDR from a human antibody that is only slightly immunoreactive with TAG-72. In particular, such variants have a relative binding affinity that is about 300 fold less than that of  
CC49.

30 CDR variants in which L-CDR3, H-CDR1, or H-CDR3 are replaced by a corresponding CDR from a human antibody do not appear to retain any binding affinity for TAG-72.

Immunogenicity of CDR Variants

The CDR variants that have a reduced immunogenicity when compared to HuCC49 formed by grafting all six (three heavy chain and three light chain) CDR from CC49 onto the variable light (V<sub>L</sub>) and variable heavy (V<sub>H</sub>) frameworks of the human MAbs LEN and 21/28'CL, respectively. That is, the CDR variants of the invention are less likely to elicit an anti-idiotypic or HAMA response. Immunogenicity can be characterized using competition radioimmunoassays known in the art in which an "anti-CC49" antibody that recognizes the parental CC49 is exposed to both the parental MAb and the variant. Generally, a reduction in immunogenicity is reflected by a reduction in binding of the variant by the anti-CC49 antibody.

CDR variants in which L-CDR1 or L-CDR2, or both, of CC49 are replaced by a corresponding CDR from a human antibody show a slight reduction in immunogenicity, that is, the variants do not bind to the anti-CC49 antibody as well as HuCC49.

CDR variants in which L-CDR3 or H-CDR2 of CC49, is replaced by a corresponding CDR from a human antibody show a substantial reduction in immunogenicity. However, the inventors have found that such variants also show a substantial reduction in immunoreactivity.

CDR variants in which H-CDR1 or H-CDR3 or CC49 are replaced by a corresponding CDR from a human antibody do not show any measurable change in immunogenicity.

SDR Variants

The inventors have discovered that all six CDR of CC49 need not be present in their entirety for the humanized antibody to retain activity. Only residues that are directly involved in antigen contact, the Specificity Determining Residues (SDRs), are needed. SDR variants are formed by replacing at least one SDR of CC49 in HuCC49 with a residue at a corresponding position from a human antibody.

It should be noted that not all CDRs include SDRs. For example, it was determined that L-CDR1 and L-CDR2 of CC49 do not have any SDRs. Therefore, in one variant of the invention, L-CDR1 and L-CDR2 are replaced entirely with human CDRs. However, SDR variants can be formed by replacing residues within these CDRs with a corresponding human residue. L-CDR1 from CC49 and LEN differ at three positions, 27b, 27f and 29. Because residues 27b, 27f, 29 are not

important for the binding affinity of CC49, a suitable SDR variant can include a corresponding human residue at any of these position, or at any combination of these positions. L-CDR2 from CC49 and LEN differ at position 53 only. Residue 53 is not considered important for the binding affinity of CC49. Thus, a suitable variant can include a corresponding human residue at position 53.

L-CDR3 of CC49 differs from LEN at three positions, 94, 96 and 97. The partially buried residue at position 97 is not important for the antigen binding activity of CC49. Thus, a suitable SDR variant can include a corresponding human residue at position 97. However, positions 94 and 96 of L-CDR3 are involved in ligand contact. and should not be replaced to generate a functional SDR variant.

H-CDR1 of CC49 and 21/28'CL differ at three positions, 31, 32 and 34. However, SDR variants which include a corresponding human residue at positions 32 and 24 demonstrate no antigen binding affinity. Thus, a functional SDR variant should not include a corresponding human residue at either of these positions.

H-CDR2 of CC49 differs from human MAb 21/28'CL at eleven positions. The residues at positions 60, 61, 62 and 64 are not required for antigen binding activity. Therefore, a SDR variant of the invention can include a corresponding human residue at any of positions 60, 61, 62 and 64, or any combinations thereof.

Generally, H-CDR3 does not need to be considered when designing an SDR variant, because it does not show any reactivity to patients' sera.

In a preferred embodiment, the variant includes a combination of CDR and/or SDR substitutions to generate a variant having reduced immunogenicity and a binding affinity that is substantially similar to that of parental CC49. Suitable combinations include CDR variants in which both L-CDR1 and L-CDR2 of CC49 are replaced by a corresponding CDR from a human antibody. Other suitable variants include a combination of SDR and CDR substitutions. For example, a suitable variant can include corresponding human residues at position 97 of the light chain in addition to a substitution of L-CDR1 and/or L-CDR2 from CC49 with the corresponding CDRs from a human antibody. In another preferred embodiment, the variant includes a substitution at position 97 on the light chain in combination with substitutions at positions 60, 61, 62 and 64 on the heavy chain. In yet another embodiment, the variant includes a substitution at position 97 on the light chain in addition to a substitution of L-CDR1 and/or L-CDR2 from CC49 with the corresponding CDRs from a human antibody in combination with substitutions at positions 60, 61, 62 and 64 on the heavy chain.

In addition to variants specifically described herein, other "substantially homologous" modified immunoglobulins can be readily designed and manufactured using various recombinant DNA techniques well known to those skilled in the art. For example, the framework regions can be varied at the primary structure level.

- 5 Moreover, a variety of different human framework regions may be used singly or in combination as a basis for the variant. In general, modifications of the genes may be readily accomplished by a variety of well-known techniques, such as site-directed mutagenesis.

- 10 Alternatively, polypeptide fragments comprising only a portion of the primary antibody structure may be produced wherein the fragment substantially retains the immunoreactive properties of the variant. These polypeptide fragments include fragments produced by proteolytic cleavage of intact antibodies by methods well known in the art, or fragments produced by inserting stop codons at the desired locations nucleotide sequence using site-directed mutagenesis. For example, a stop  
15 codon can be inserted after C<sub>H</sub>1 to produce Fab fragments or after the hinge region to produce F(ab')<sub>2</sub> fragments. Single chain antibodies and fusion proteins which includes at least an immunoreactive fragment of the variant are also included within the scope of the invention. For example, the variants may be directly or indirectly attached to effector moieties having therapeutic activity. Suitable effector moieties  
20 include cytokines, cytotoxins, radionuclides, drugs, immunomodulators, therapeutic enzymes, anti-proliferative agents, etc. Methods for attaching antibodies to such effectors are well known in the art.

#### Binding Affinity of SDR Variants

- 25 L-CDR1 from CC49 and LEN differ at three positions, 27b, 27f and 29. Since L-CDR1 of CC49 can be replaced with the corresponding CDR from LEN without any significant loss of antigen binding reactivity, residues 27b, 27f, 29 are not considered important for the binding affinity of CC49. Thus, a variant of the invention can include a corresponding human residue at any of these three positions,  
30 or any combination thereof, and retain a binding affinity that is substantially similar to that of the parent HuCC49.

- In L-CDR2, CC49 and LEN differ at position 53 only. Since L-CDR2 of CC49 can be replaced with the corresponding CDR from LEN without any significant loss of antigen binding reactivity, residue 53 is not considered important  
35 for the binding affinity of CC49. Thus, the humanized antibody of the invention

can include a corresponding human residue at residue 53 and retain a binding affinity that is substantially similar to that of the parent HuCC49.

L-CDR3 of CC49 differs from LEN at three positions, 94, 96 and 97. The partially buried residue at position 97 is not important for the antigen binding activity of CC49. Thus, the humanized antibody of the invention can include a corresponding human residue at position 97 and retain a relative binding affinity that is substantially similar to that of CC49. However, positions 94 and 96 of L-CDR3 appear to be involved in ligand contact. Therefore, an SDR variant which includes a corresponding human residue at either position 94 or 96, or both will generally suffer total or near total loss of antigen binding reactivity.

H-CDR1 of CC49 and 21/28'CL differ at three positions, 31, 32 and 34. SDR variants which include a corresponding human residues at positions 32 and 24 demonstrate no antigen binding affinity.

H-CDR2 of CC49 differs from human MAb 21/28'CL at eleven positions. The residues at positions 60, 61, 62 and 64 do not appear to be required for antigen binding activity. Therefore the humanized antibody of the invention can include a corresponding human residue at any of positions 60, 61, 62 and 64, or any combinations thereof, and the variant will retain a binding affinity that is substantially similar to that of CC49.

#### Immunogenicity of SDR Variants

SDR variants are particularly beneficial because some CDRs that are important for immunoreactivity are also immunogenic (e.g., L-CDR3 and H-CDR2). Thus, the immunogenicity of various SDR replacements within L-CDR3 and H-CDR2 were examined.

As shown in Figure 2, L-CDR3 consists of residues 89-97 and H-CDR2 consists of residues 50-65. The inventors have found that SDR variants which include a corresponding human residue in positions 32 and 34 (found within H-CDR1) or at position 97 (found within L-CDR3) are still immunogenic. Whereas, SDR variants which include a corresponding human residue in positions 60, 61, 62, and 64 (found within H-CDR2) or at position 94 (found within L-CDR3) show a reduction in immunogenicity. SDR variants which include a corresponding human residue in position 96 (found within L-CDR3) do not appear to be immunogenic.

Generally, the residues found in H-CDR3 does not need to be considered when designing SDR variants, because it does not show any reactivity to patients' sera.

### 5 Human Antibodies

Suitable human antibodies include, but are not limited to: ROY, AU, REI, HAU, HK101'CL, SCW, WEA, HK137'CL, HK134'CL, DAUDI'CL, WALKER'CL, GAL(1), LAY, WES, Vb'CL, HK102'CL, EU, DEN, AMYLOID BAN, MEV, Vd'CL, Va'CL, KUE, Ve'CL, V13'CL, V18A'CL, V19A'CL, V19B'CL, V18C'CL, 10 NIM, CUM, GM603CL, FR, RP M1-6410'CL, TI, WOL, SIE, NG9'CL, NEU, GOT, PAY, SON. GAR', PIE, FLO, GLO, CUR, IARC/BL41'CL, POM, REE, K-EV15'CL, VJI'CL, VKAPPAIV, GERMLINE'CL, PB171'CL, LEN, NEWM, HA, NIG-64, NEW, BL2'CL, WAH, NIG-77, VOR, RHE, LOC, OKA, COX, NIG-51, NIG-84, MES, WH, NEI, WEIR, TOG, TRO, BOH, NIG-58, VIL, WIN, 41'CL, 15 HIL, LAP, GAR, MOT, BO, MDG, AMYLOID-AR, SUT, THO, LBV'CL, NIG-48, HG3'CL, ND'CL, COR, DAW, OU, MCE', CE-1'CL, HE, SUP-T1, VH-JA'CL, HIG1'CL, TUR, LAMDA-VH26'CL, WAS, H11'CL, TEI, BRO'IGM, GRA', ZAP, JON, DOB, NIE, 333'CL, 1H1'CL, 1B11'CL, 126'CL, 112'CL, 115'CL, KOL and 21/28'CL. New human antibodies are being discovered and sequenced, many of 20 those, as of yet unknown antibodies may also be suitable. Preferably, human antibody has a sequence that is identical or substantially similar (containing as few mutations as possible) to the human germ line sequences. For example, the light chain CDR of CC49 in HuCC49 can be replaced with the corresponding CDR from LEN (Kabat et al., 1991) and the heavy chain CDR can be replaced with the 25 corresponding CDR from 21/28'CL (Kabat et al., 1991).

### Methods of Producing

The variants of the invention can be produced by expressing the appropriate DNA sequence in a host after the sequence has been operably linked to (i.e., 30 positioned to ensure the functioning of) an expression control sequence. Such expression vectors are typically replicable in a host organism either as episomes or as an integral part of the host chromosomal DNA. The expression vectors typically contain expression control sequences compatible with the host cell, such as an origin of replication. In addition, the expression vector will typically include a promoter. 35 Suitable promoters include the polyhedrin promoter, lactose promoter system, a

tryptophan promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. Promoters typically control expression of the gene, optionally, with operator sequences, and have ribosome binding site sequences and the like for initiating and completing transcription and translation. Commonly, expression vectors will contain selection markers. DNA sequences encoding the light chain and heavy chain of the antibody may be inserted into separate expression vectors, or into the same expression vector.

Suitable hosts include prokaryotic strains such as *E. coli*; *Bacilli*, including *Bacillus subtilis*; enterobacteriaceae, including *Salmonella*, *Serratia* and *Psuedomonas*. Suitable hosts also include eukaryotic hosts such as yeast, including *Saccharomyces*; *Pichia pastoris*; Sf9 insect cells; Sp2/0, VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines; W138, BHK, COS-7 and MDCK cell lines.

The vectors containing the DNA segments of interest can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection, calcium phosphate treatment, electroporation or cationic liposome mediated transfection (such as DOTAP). Successfully transformed cells, can be identified by a variety of techniques well known in the art for detecting the binding of a receptor to a ligand.

Once expressed, the gene products can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, and gel electrophoresis. Substantially pure immunoglobulins of at least about 90% to about 95% homogeneity are preferred, and 98% to 99% or more homogeneity most preferred for pharmaceutical uses.

#### Methods of Use

Once purified, the variants of the invention may be used therapeutically, or in developing and performing assays, *in vivo* or *in vitro* diagnostic procedures, and imaging. The variants of the invention are particularly useful for the treatment of diseases such as cancer, in particular for treating or detecting cancer. The variants can be administered to a patient alone or in combination with a pharmaceutical formulation. Typically, the variants are incorporated into a pharmaceutically acceptable, non-toxic, sterile carrier as a suspension or solution. The antibodies of the invention can be used as separately administered compositions or given in conjunction with chemotherapeutic or immunosuppressive agents.

The variants provide unique benefits when used for the treatment of cancer. In addition to the ability to bind specifically to malignant cells and localize tumors without binding to non-cancerous cells, the variants have a reduced immunogenicity when compared to HuCC49.

5 For diagnostic purposes, the antibodies may either be labeled or unlabeled. Unlabeled antibodies can be used in combination with other labeled antibodies (second antibodies) that are reactive with the humanized antibody, such as antibodies specific for human immunoglobulin constant regions. Alternatively, the antibodies can be directly labeled. A wide variety of labels can be employed, such  
10 as radionuclides, fluors, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, ligands (particularly haptens), etc. Numerous types of immunoassays are available and are well known to those of skill in the art.

Kits according to the present invention include frozen or lyophilized variant to be reconstituted by thawing or by suspension in a liquid vehicle. The kits may  
15 also include a carrier or buffer. Preferably, the kit also comprises instructions for reconstituting and using the variant antibody.

### **Working Examples**

To identify the CDRs essential for binding, a panel of variant HuCC49 MAbs  
20 were generated using the baculovirus expression system. HuCC49 was prepared by grafting MAb CC49 CDRs onto the V<sub>L</sub> and V<sub>H</sub> frameworks of the human MAbs LEN and 21/28' CL, respectively, as described by Kashmiri et al., (1995) Hybridoma, 14:461-473. Six CDR variants were constructed by replacing a single  
25 CC49 CDR of either the light or heavy chain with the corresponding human antibody CDR (LEN and 21/28'CL, respectively). Variants were denoted as L-1, L-2, L-3, H-1, H-2 or H-3. A seventh variant, L-1,2 was made by replacing two  
CC49 light chain CDRs (L-CDR1 and L-CDR2) with the corresponding CDRs of the human antibody LEN.

Since the seven CDR variants were derived by simply replacing the murine  
30 CDRs with the human antibody hypervariable regions, all of the variants carry identical V<sub>H</sub> and V<sub>L</sub> frameworks and  $\gamma$ 1 and  $\kappa$  chain constant regions

SDR heavy chain and light variants were constructed by substituting mutagenic nucleotides in or near the CDRs.



**Example I: Preparation of CDR Substituted MAb CC49**

According to the invention, CDR variants are formed by replacing at least one CDR of CC49 in HuCC49 with a corresponding CDR from a human antibody.

5 The CDR variants of the invention include:

- Variant L-1: L-CDR1 of CC49 was replaced with that of LEN.
- Variant L-2: L-CDR2 of CC49 was replaced with that of LEN.
- Variant L-3: L-CDR3 of CC49 was replaced with that of LEN.
- Variant L-1,2: L-CDR1 and L-CDR2 of CC49 were replaced with that of LEN.
- 10 • Variant H-1: H-CDR1 of CC49 was replaced with that of 21/28'CL.
- Variant H-2: H-CDR2 of CC49 was replaced with that of 21/28'CL.
- Variant H-3: H-CDR3 of CC49 was replaced with that of 21/28'CL.

*Production of Oligomers to generate V<sub>H</sub> Variants*

15 Synthesis of three variant V<sub>H</sub> genes was performed using the overlap extension PCR technique described by Kashmiri et al., (1995) Hybridoma 14:461-473. Four 124-137 base pair long overlapping oligonucleotides, (which together encompass the entire sequence of the variant V<sub>H</sub> gene on alternating strands) were used to generate variant V<sub>H</sub> genes. (Figure 12 B) The oligomers were supplied by  
20 Midland Certified Reagent Co., Midland, TX. Instead of a template DNA, the PCR mixture contained 2 pmoles of the four oligonucleotides. PCR was carried out by three cycles of a denaturing step at 94°C for 1 minute, a primer annealing step at 55°C for 2 minutes, and an extension step at 70°C for 2 minutes, followed by 17  
25 additional cycles of denaturation (94°C, 1 minute), primer annealing (55°C, 2 minutes), and extension (72°C, 1 minute). All polymerase chain reactions (PCRs) were carried out in a final volume of 100 µl of PCR buffer containing 100 µM of dNTPs, 5 units of Taq DNA polymerase (Boehringer Mannheim) and 20 pmol of each end primer.

30 *Production of Oligomers to Generate V<sub>L</sub> Variants*

The three variant V<sub>L</sub> genes were generated using 30-43 base oligonucleotides as a mutagenic primer. The oligonucleotides contained the desired base changes in the targeted CDR. The mutagenic primers for the V<sub>L</sub> genes were synthesized using a Model 8700 DNA synthesizer (Miligen/Bioresearch, Burlington,  
35 VT). (Figure 12 A) Primer induced mutagenesis was carried out by a two-step PCR

method, as described by Landt et al., (1990) Gene, 96:125–128.

pLNXCHuCC49HuK (Kashmiri et al, (1995) Hybridoma 14:461–473) (Figure 2) was used as a template in both steps. In the first step, the mutagenic primer was used as a 3' primer while a 20 nucleotide long end primer served as a 5' primer. The product of the first PCR was gel purified and utilized as a 5' primer for the second PCR in which a 20 nucleotide long end primer was used as a 3' primer. The 20 nucleotide long end primers used for DNA amplification were supplied by Midland Certified Reagent Co. (Midland, TX). The sequences for these primers are reported by Kashmiri et al., (1995) Hybridoma 14:461–473 and are as follows:

1. 5' V<sub>H</sub>, 5'-CTA AGC TTC CAC CAT GGA G-3'
2. 3' V<sub>H</sub>, 5'-ATG GGC CCG TAG TTT GGC G-3'
3. 5' V<sub>L</sub>, 5'-GCA AGC TTC CAC CAT GGA TA-3'
4. 3' V<sub>L</sub>, 5'-AGC CGC GGC CCG TTT CAG TT-3'

Each of the primers carries a single restriction endonuclease site at its flank. The 5' primers carry a *Hind*III site, while the 3' V<sub>H</sub> primer carries an *Ap*I, and the 3' V<sub>L</sub> primer has a *Sac*II site. The restriction endonuclease recognition sequences are underlined.

The first PCR was carried out in a final volume of 100 µl containing 10 ng of the template DNA, 20 pmol each of the 3' and 5' primers, 100 µM dNTPs and 5 units of Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN). Each step of the PCR consisted of 25 cycles of denaturation (94°C, 1 minute), primer annealing (45°C, 2 minutes), and extension (72°C, 2 minutes). The PCR product was extracted with phenol/chloroform, precipitated with ethanol and gel purified prior to insertion into a vector.

#### Example II. Assembly of CDR Substituted MAb CC49 PCR Products

The PCR products encoding the V<sub>H</sub> were treated with *Hind*III/*Ap*I. The PCR products were subcloned for sequencing in pBluescript S/K+ (Stratagene, La Jolla, CA) at a *Hind*III/*Ap*I site after the plasmid was linearized using the appropriate restriction endonucleases. Inserts were sequenced to check their fidelity to their templates.

To assemble the variable and constant regions of the heavy chain the *Hind*III/*Ap*I insert was released from pBluescript. A DNA fragment encoding the human γ1 constant region was excised from pLgpCXHuCC49HuG1 (Kashmiri et al,

(1995) *Hybridoma* 14:461–473), (Figure 3) by *ApaI/ClaI* cleavage. The *HindIII/ApaI* and the *ApaI/ClaI* fragments were joined. The recombinant was unidirectionally inserted, by three way ligation, between the *HindIII* and *ClaI* sites of pBluescript. The DNA sequence encoding the entire heavy chain was then  
5 cleaved from pBluescript by *HindIII/ClaI* digestion. Its termini were filled in using the Klenow fragment of the DNA polymerase. The insert was subcloned in a light chain construct of pAcUW51 (Figure 4), at the blunt ended *BamHI* site located downstream of the polyhedrin promoter.

The PCR products encoding the  $V_L$  were treated with *HindIII/SacII*. The  
10 PCR products were subcloned for sequencing in pBluescript S/K+ (Stratagene, La Jolla, CA) at a *HindIII/SacII* site after the plasmid was linearized using the appropriate restriction endonucleases. Inserts were sequenced to check their fidelity to their templates.

To assemble the variable and constant region of the light chain, the  
15 *HindIII/SacII* insert was released from the pBluescript construct. A DNA fragment encoding the human kappa constant region was excised from pLNCXHuCC49HuK (Kashmiri et al, (1995) *Hybridoma* 14:461–473), (Figure 2) by *SacII/ClaI* treatment. The *HindIII/SacI* fragments were joined to the *HindIII/ClaI* linearized pBluescript by three way ligation. The entire light chain was cleaved from pBluescript using  
20 *EcoRI*. The *EcoRI* fragment was inserted into the baculovirus expression vector pAcUW51 (Pharmingen, San Diego, CA) at the *EcoRI* site located downstream from the p10 promoter.

The baculovirus expression construct of the parental HuCC49 was generated using DNA fragments encoding HuCC49 heavy and light chains obtained from  
25 PLNCXHuCC49HuK and pLgpCXHuCC49HuG1. PLNCXHuCC49HuK was cleaved with *HindIII*. The resulting ~1.0 Kb DNA fragment encoding HuCC49HuK was subcloned in pBluescript at the *HindIII* site. The resulting construct was then cleaved with *BamHI* and the fragment was cloned in the baculovirus vector pAcUW51 at the *BamHI* site, downstream from the polyhedrin promoter. A ~1.4  
30 Kb DNA fragment encoding HuCC49HuG1 was cloned from pLgpCXHuCC49HuG1 using *HindIII/ClaI*. The DNA fragment was filled using the Klenow fragment of DNA polymerase. pAcUW51 was linearized with *BglII* and its ends blunted using the Klenow fragment. The DNA fragment was then inserted in the pAcUW51 expression construct of HuCC49HuK, downstream from the p10  
35 promoter.

Example III. Generation of Baculovirus Recombinant CDR Substituted CC49 MAb

5 Serum free adapted Sf9 insect cells (Gibco BRL, Gaithersburg, MD) were cultured at 28°C in Sf900-II medium (Gibco BRL) without supplements as described by Salgaller et al, (1993) Cancer Res., 53:2154-2161. To develop the recombinant baculovirus,  $1 \times 10^6$  Sf9 cells in a 35 mm dish were co-transfected with 0.5 ml pAcUW51 derived baculovirus expression construct of the CDR substituted  
10 light chain gene and the HuCC49 heavy chain gene along with linearized BACULOGOLD wild type baculovirus DNA (Pharmingen), using a cationic liposome mediated transfection system, DOTAP (Boehringer Mannheim) according to the suggested protocol. Similarly, variant antibodies containing CDR substituted heavy chain were produced by co-transfecting Sf9 cells with BACULOGOLD  
15 baculovirus DNA and baculovirus dual expression constructs carrying CDR substituted heavy chain and HuCC49 light chain genes. Baculovirus recombinant HuCC49 (hereafter referred to as HuCC49) was used as a control antibody. HuCC49 was produced by transfecting insect cells with pAcUW51 carrying HuCC49 light and heavy chains.

20 Five days after transfection, the infectious supernatants were harvested from the transfectants. 1 ml of this supernatant was serially diluted and used to infect a monolayer of  $5 \times 10^6$  Sf9 cells in a 100 mm dish. The cells were then overlaid with 0.5% Baculovirus Agarose (Invitrogen, Carlsbad, CA) as described by Bei et al., (1995) J. Immunol. Methods, 186:245-255. Viral plaques were expanded by three  
25 rounds of infection. For each round of expansion, a larger population of freshly seeded monolayers of Sf9 cells were infected, using the highest producing clone as a source of inoculum. The putative recombinant viral plaques were purified and isolated in 1 ml of Sf900 media. If necessary, viruses were further amplified by infecting cells at an Multiplicity of Infection (MOI) of 0.1. To produce the  
30 recombinant antibodies,  $6.0 \times 10^8$  Sf9 cells were infected with the infectious supernatant at an MOI of 5.

Purification of CDR Substituted MAbs

The culture supernatant was clarified by pelleting cell debris at 10,000xg,  
35 and was applied to an ion-exchange column (DE52; Whatman, Hillsboro, OR) at pH

7.2 to remove extraneous proteins. The unbound protein fraction was subjected to protein G (Gibco BRL) affinity chromatography. The material bound to protein G was eluted from the column using 0.1 M glycine hydrochloride buffer, pH 2.6 and the pH of the eluted material was immediately adjusted to 7.4 using 1.0 M Tris  
5 buffer, pH 8.0. The buffer was replaced by phosphate buffered saline and the eluted material was concentrated using a Centricon 30 micro concentrator (Amicon, Beverly, MA). Protein concentration was determined by the method of Lowry et al., (1951) J. Biol. Chem. 193:265–275. The purity of the antibody preparation was analyzed using a precast continuous 4–15% SDS–polyacrylamide Tris–glycine gel  
10 (Novex Systems, San Diego, CA) and visualized by Coomassie blue staining as described by Kashmiri et al., (1995) Hybridoma 14:461–473.

#### Radiolabeling of MAbs

The murine MAb CC49 and HuCC49 were labeled with Na<sup>125</sup>I using the  
15 iodogen (Pierce, Rockford, IL) method as described by Fraker (1978) Biochem. Biophys. Res. Commun., 80:849–857 and Colcher (1988) Cancer Res., 48:4597–4603. The protocol routinely resulted in specific activities at 5–10 µCi/µg. The immunoreactivities of the radiolabeled MAbs were assessed by the radioimmunoassay described by Schott et al., (1992) Cancer Res., 52:6413–6417  
20 using bovine submaxillary mucin (BSM) immobilized on a solid support (Reacti-gel HW 65F; Pierce)

#### Immunoglobulin Production

The titer of the transfectants and the putative viral plaques were assayed for  
25 immunoglobulin production by enzyme–linked immunosorbent assay (ELISA) based on reactivity of the test aliquot with goat anti–human Fc (γ1) and goat anti–human kappa antibodies as described by Bei et al., (1995) J. Immunol. Methods, 186:245–255. Transfectants and viral plaques derived from each of the expression constructs were positive for immunoglobulin production.

30 However, when the transfectants and the viral plaques were assayed for immunoreactivity with TAG–72 positive bovine submaxillary mucin (BSM), the clones derived from the expression constructs carrying L–1, L–2 and L–1,2 were positive, while those generated by the H–2 expression construct were barely immunoreactive. Those derived from the constructs carrying either L–3, H–1 and  
35 H–3 demonstrated no immunoreactivity with BSM at all.

It was then assessed whether the poor or lack of BSM reactivity of the clones derived from L-3, H-1, H-2 and H-3 expression constructs was due to low levels of immunoglobulin secretion by these clones. To that end, Sf9 cells were infected with the infectious supernatants at an MOI of 5 and cultured under the conditions described above. The secreted antibody was purified from equal volumes of the culture supernatant from each of the infected cultures, and analyzed by SDS-PAGE. The gel profile under non-reducing conditions showed that the mobility of the variant antibodies was identical to that of the HuCC49, which has a molecular weight of approximately 160 kDa (data not shown). Under reducing conditions, the variant antibodies, like the HuCC49 MAb, yielded two protein bands of approximately 25-28 kDa and 50-55 kDa (Figure 6). These mobilities are in conformity with the molecular masses of the immunoglobulin heavy and light chains. More importantly, it is evident that regardless of their BSM reactivity, clones derived from each of the constructs encoding CDR-substituted heavy or light chain produce as much immunoglobulin as the clone derived from the constructs encoding the parental humanized heavy and light chains.

Example IV. Competition Radioimmunoassays for CDR Substituted Variants

Binding Affinity of Variant Antibodies

The relative binding affinity of the HuCC49 and the CDR substituted variant antibodies to TAG-72 was determined using the competition radioimmunoassay (RIA) described by Milenic et al., (1991) Cancer Res., 51:6363-6371. Serial dilutions of the purified variant MAbs, as well as the parental HuCC49, were prepared in phosphate buffered saline (PBS) containing 1 % bovine serum albumin (BSA). 25  $\mu$ l was added to the wells of microtiter plates containing 10 ng BSM.  $^{125}$ I-labeled HuCC49 (50,000 cpm in 25  $\mu$ l) was then added to each well. The plates were incubated overnight at 4°C and then washed and counted in a  $\gamma$ -scintillation counter.

Unlabeled HuCC49 or its variants were used to compete for the binding of  $^{125}$ I-HuCC49 to TAG-72 positive BSM. The variants, L-1, L-2 and L-1,2, were found to completely inhibit the binding of the  $^{125}$ I-labeled HuCC49 to TAG-72, while L-3 did not compete at all (Figure 7).

The relative affinity constants were calculated by the modification of the Scatchard method described by Frankel et al., (1979) Mol. Immunol., 16:101-106.

An approximation of the specific activity of the  $^{125}\text{I}$ -HuCC49 was made and used to determine the final concentration for each of the dilutions of the variant MABs. The calculations were performed as described by Milenic et al., (1991) Cancer Res., 51:6363-6371.

5 The relative affinity constants ( $K_a$ ) of the variants were as follows:

- L-1 had a  $K_a$  of  $3.3 \times 10^{-8}$  M (only about 2-fold less than that of HuCC49).
- L-2 had a  $K_a$  of  $6.81 \times 10^{-8}$  M (comparable to that of HuCC49).
- L-1,2 had a  $K_a$  of  $2.9 \times 10^{-8}$  M (only about 2-fold less than that of HuCC49).
- H-1 and H-3 displayed no competition
- 10 • H-2 competed only slightly with the HuCC49. The  $K_a$  of H-2 was  $0.018 \times 10^{-8}$  M (approximately 300-fold less than the  $K_a$  of HuCC49).

Reactivity of the CC49 anti-idiotypic antibodies to the variant antibodies

The variant MABs were also characterized in the competition  
15 radioimmunoassay RIA described by Irvine et al., (1993) Cancer Immunol.  
Immunother., 36:281-292 using mouse anti-idiotypic MAB generated against MAB  
CC49. Three anti-idiotypic (AI49-8, AI49-3 and AI49-1) were selected,  
representing each of the anti-idiotypic subsets,  $\alpha$ ,  $\beta$ , and  $\gamma$ , respectively. In the same  
manner described above, 100 ng of MAB AI49-3 ( $\beta$ -subset), AI49-1 ( $\gamma$ -subset) or  
20 AI49-8 ( $\alpha$ -subset) were adsorbed to each well of a 96-well microtiter plate. 25  $\mu\text{l}$   
of the serially diluted variant MABs or HuCC49 was added to each well along with  
25  $\mu\text{l}$  of  $^{125}\text{I}$ -murine CC49. The plates were washed and counted after an overnight  
incubation at 4°C.

The results for the light chain variants are shown in Figure 8. For the AI49-  
25 3 ( $\beta$ -subset): L-CDR1 appears to be only partially involved in the recognition of  
CC49 by AI49-3; L-CDR2 does not appear to be involved in the recognition of  
CC49 by AI49-3; and L-CDR3 appears to be important for recognition of CC49 by  
AI49-3. For the AI49-1 ( $\gamma$ -subset): L-CDR1 appears to be not required for  
recognition of CC49 by AI49-1; L-CDR2 appear to be only modestly involved in  
30 the recognition of CC49 by AI49-1; and L-CDR3 appears to be important for  
recognition of CC49 by AI49-1. For the AI49-8 ( $\alpha$ -subset): neither L-CDR1, L-  
CDR2, nor L-CDR3 appear to have any influence on the interaction of AI49-8 with  
CC49.

The results for the heavy chain variants are shown in Figure 9. For the  
35 AI49-3 ( $\beta$ -subset): H-CDR1 and H-CDR3 do not appear to be involved in binding

of HuCC49 to AI49-3, while H-CDR2 appears to be important for recognition of CC49 by AI49-3 (approximately 4-15 times more competitor is required for 50 % inhibition by H-2 as compared to HuCC49). For the AI49-1 ( $\gamma$ -subset): H-CDR1 and H-CDR3 do not appear to be involved in binding of HuCC49 to AI49-1, while  
5 H-CDR2 appears to be important for recognition of CC49 by AI49-1 (approximately 4-15 times more competitor is required for 50 % inhibition by H-2 as compared to HuCC49). For the AI49-8 ( $\alpha$ -subset): H-CDR1 and H-CDR3 do not appear to be involved in binding of HuCC49 to AI49-8, while H-CDR2 appears to be important for recognition of CC49 by AI49-8 (there is a complete loss of  
10 inhibition by the variant).

An analysis of patient reactivity to the variants of HuCC49 show that three of the 6 CDRs (L-CDR2, H-CDR1 and H-CDR3) do not seem to be recognized by the patient, while L-CDR1 and H-CDR2 appear to be involved in the patient's recognition of HuCC49 to some degree. L-CDR3 (which is important for antigen  
15 binding) is the immunodominant CDR recognized by the patient. L-CDR3 is immunodominant in mice as well (AI49-1 and AI49-3, the two anti-idiotypic antibodies that inhibit antigen binding of HuCC49, require L-CDR3 for recognition of HuCC49).

#### 20 Example V. High Performance Liquid Chromatography

The CDR variants were further characterized using the serum from a patient that had received  $^{177}\text{Lu}$ -CC49 in a phase 1 radioimmunotherapy clinical trial (Mulligan et al., (1995) Clin. Cancer Res., 1:1447-1454. Several of the patients in  
25 this study were found to have anti-idiotypic antibodies to MAb CC49. One patient was selected to perform a preliminary study to identify whether any of the CC49 CDRs were immunodominant.

Using a modification of the method reported by Colcher et al., (1990) J. Nucl. Med., 31:1133-1142 and Mulligan et al., (1995) Clin. Cancer Res., 1: 1447-  
30 1454, serial dilutions of the purified CDR variants were incubated with the patient's sera along with  $^{125}\text{I}$ -labeled HuCC49. Specifically, the method of Colcher and Mulligan was modified as follows: prior to the study, HAMA and TAG-72 were removed from the sera by adsorption with CC92 conjugated solid support. The amount of sera required for half maximal complex formation with HuCC49 was then  
35 determined. Specifically, 8  $\mu\text{l}$  of patient sera was mixed with  $\sim 500,000$  cpm of  $^{125}\text{I}$ -



HuCC49 and serial dilutions of purified HuCC49 or its variants. The preparations were brought to a final volume of 50  $\mu$ l.

The ability of the variants to inhibit complex formation of the patent sera with  $^{125}$ I-labeled HuCC49 was monitored using HPLC analysis. 25  $\mu$ l of each solution was applied to a TSK3000 analytical column (7.8 mm x 30 cm; Tosohaas, 5 Montgomeryville, PA) and eluted at 0.5 ml/min with 100 mM KCl in 67 mM sodium phosphate (pH 6.8). Radioactivity was monitored using a flow-through  $\gamma$ -scintillation detector (Model 170, Beckman).

If the variant contained the CDR recognized by the patient, then the variant 10 would compete with the radiolabeled HuCC49 and complex formation would not occur and there would not be an alteration in the retention time of the  $^{125}$ I-HuCC49. If the variant no longer contained a CDR recognized by the patient, then complex formation would result. Thus, the ability of the CDR variants to inhibit complex formation of the patient sera with the radiolabeled HuCC49 was determined by the 15 retention time of the  $^{125}$ I-HuCC49. The percent inhibition of complex formation was calculated and plotted versus concentration of each competitor to evaluate the degree of the patient's reactivity with the individual CDR variants. Figure 15 shows a comparison of patient reactivity with HuCC49 and CDR variants.

- 20 • L-1 (variant without CC49 L-CDR1) showed some inability to inhibit complex formation. Thus L-CDR1 appears to be somewhat involved in immunogenicity (0.7 $\mu$ g of competitor was required for 50% inhibition of complex formation).
- L-2 appeared to compete better than parental HuCC49 by 2 fold (an enhanced recognition by the patient)
- 25 • L-3 showed no inhibition of complex formation, thus L-CDR3 appears necessary for immunogenicity
- L-1,2 demonstrated some inability to inhibit complex formation, indicating that L-CDR1 and/or L-CDR2 are somewhat involved in immunogenicity.
- H-1 inhibits complex formation and therefore contributes to immunogenicity.
- 30 • H-2 showed little complex formation, thus H-CDR2 does not appear to be necessary for immunogenicity (10  $\mu$ g of competitor was unable to achieve 50% inhibition of complex formation).
- H-3 demonstrated some inability to inhibit complex formation, thus H-CDR3 appears to be somewhat involved in immunogenicity (0.4 $\mu$ g of competitor was 35 required for 50% inhibition of complex formation).

Example VII. Preparation of SDR Substituted MAb CC49

Padlan et al., (1995) FASEB J., 9:133-139 disclose that the SDRs of light  
5 chain are bounded by positions 27d and 34; 50 and 55; and 89 and 96. The heavy  
chain SDRs are contained within positions 31 and 35b; 50 and 58; and 95 and 101.

Figure 2 shows the differences between the amino acid residues of the light  
chain CDRs of CC49 and LEN, and the heavy chain CDRs of CC49 and 21/28'CL.

In L-CDR1, CC49 and LEN differ in three residues; at positions 27b, 27f  
10 and 29. The residues at positions 27b (a buried residue) and 27f were found not to  
be directly involved in ligand contact, while the one at position 29 was found to  
interact with ligand in two complexes; in one only by main chain atoms. Residue  
27b is located outside the suggested SDR boundaries. Residues 27f and 29 are well  
within the suggested SDR boundaries.

15 In L-CDR2, CC49 and LEN differ at position 53 only, and this position was  
found to be involved in ligand contact in only three of the 31 complexes of known  
structure. Residue 53 is well within the suggested SDR boundaries.

Since L-CDR1 and 2 of CC49 were replaced with their counterparts from  
LEN without any significant loss of antigen binding reactivity (above), it was  
20 concluded that residues 27b, 27f, 29 and 53 were not important for binding of CC49  
to its antigen. L-CDR1 and L-CDR2 of CC49 were not considered for the mutation  
experiments because they were replaced with the corresponding CDRs of the human  
MAb LEN without significant loss of antigen binding reactivity.

The immunodominant L-CDR3 of CC49 differs from LEN at three  
25 positions, 94, 96 and 97. Each of the three residues of CC49 L-CDR3 was replaced  
with the residue present at the corresponding position in the LEN CDR to generate  
light chain variants <sup>94</sup>L, <sup>96</sup>L and <sup>97</sup>L, respectively. Another light chain variant, <sup>94,97</sup>L  
was generated carrying two substitutions, one at position 94 and the other at 97.  
Two additional variants were derived from the HuCC49 light chain variant L<sub>1,2</sub>, in  
30 which the L-CDR1 and L-CDR2 of CC49 were earlier replaced with their  
counterparts from the human MAb LEN. One variant, <sup>97</sup>L<sub>1,2</sub>, carried a single  
substitution at position 97. The other, <sup>94,97</sup>L<sub>1,2</sub>, had substitutions at two positions, 94  
and 97.

Of the three residues that differ between L-CDR3 of CC49 and LEN, a  
35 partially buried residue at position 97 was not important for the antigen binding

activity of CC49. This residue is not located within the suggested boundary of SDRs of the L-CDR3. Thus, variant <sup>97</sup>L did not show any loss in antigen binding activity. Variant <sup>97</sup>L<sub>1,2</sub> showed only an insignificant loss of antigen binding activity.

Positions 94 and 96 of L-CDR3 are involved in ligand contact in 19 and 22 known antibody:antigen complexes, respectively. Thus it was consistent that variants <sup>96</sup>L and <sup>94</sup>L suffered total and near total loss of antigen binding reactivity. When the mutation at position 94 was imposed on the variants <sup>97</sup>L and <sup>97</sup>L<sub>1,2</sub>, it destroyed their antigen binding function.

H-CDR1 of CC49 and 21/28'CL differ at three positions, 31, 32 and 34. The residue at position 31 is directly involved in ligand binding in 12 of the 31 complexes; in five of those, only main chain atoms were involved. The residue at position 32 is ligand contacting in eight of the 31 complexes of known structure. The residue at position 34 is involved in ligand contact in none of the 31 complexes of known structure. Residues at positions 32 and 34 of the CC49 H-CDR1 were replaced with the corresponding residues of 21/28'CL MAb (<sup>32,34</sup>H) to test whether position 32 is important for ligand contact and in eliciting anti-idiotypic response.

H-CDR2 of CC49 differs from human MAb 21/28'CL at eleven positions. The residues at positions 60, 61, 62 and 64 were not ligand contacting in any of the complexes of known structure. Therefore, these residues of CC49 were prime candidates for replacement. Accordingly, a heavy chain variant of HuCC49, <sup>60-62,64</sup>H, was generated by replacing these residues of HuCC49 with their counterparts in human MAb 21/28'CL.

H-CDR3 was not considered for mutations, because it did not show any reactivity to patient's sera (above).

The following SDR variants were made:

- Variant <sup>94</sup>L: residue 94 of CC49 L-CDR3 was replaced with the residue present at the corresponding position in LEN.
- Variant <sup>96</sup>L: residue 96 of CC49 L-CDR3 was replaced with the residue present at the corresponding position in LEN.
- Variant <sup>97</sup>L: residue 97 of CC49 L-CDR3 was replaced with the residue present at the corresponding position in LEN.
- Variant <sup>94,97</sup>L: residue 94 and 97 of CC49 L-CDR3 was replaced with the residue present at the corresponding position in LEN.
- Variant <sup>97</sup>L<sub>1,2</sub>: derived from the HuCC49 light chain variant L<sub>1,2</sub>, in which the L-CDR1 and L-CDR2 of CC49 were replaced with their counterparts from the

human MAb LEN; residue 97 of CC49 L-CDR3 was replaced with the residue present at the corresponding position in LEN.

- Variant <sup>94,97</sup>L<sub>1,2</sub>: derived from the HuCC49 light chain variant L<sub>1,2</sub>, in which the L-CDR1 and L-CDR2 of CC49 were replaced with their counterparts from the human MAb LEN; residues 94 and 97 of CC49 L-CDR3 were replaced with the residue present at the corresponding position in LEN.
- Variant <sup>32,34</sup>H: residues at positions 32 and 24 of the CC49 H-CDR1 were replaced with the corresponding residues of 21/28'CL MAb.
- Variant <sup>60-62,64</sup>H: residues at positions 60, 61, 62 and 64 of the CC49 H-CDR1 were replaced with the corresponding residues of 21/28'CL MAb.

#### Production of Oligomers

The oligomers were produced essentially as described in Example 1.

pLgpCXHuCC49H<sub>u</sub>Y1, the expression construct for parental HuCC49 heavy chain genes was used as the template for heavy (<sup>32,34</sup>H and <sup>60-62,64</sup>H) chain variant gene synthesis. pLNCXHuCC49H<sub>u</sub>K, the expression construct of the parental HuCC49 light chain gene was used as a template for the light (<sup>94</sup>L, <sup>96</sup>L, <sup>97</sup>L and <sup>94,97</sup>L) chain variant gene synthesis. Variants L<sub>1</sub> and L<sub>1,2</sub> were developed by replacing only the L-CDR1 or both L-CDR1 and L-CDR2 of CC49, respectively, with their LEN counterparts. For the synthesis of <sup>94</sup>L<sub>1,2</sub> and <sup>94,97</sup>L<sub>1,2</sub> genes, an expression construct of the L<sub>1,2</sub> variant in a baculoviral expression construct was used as a template.

Mutagenic oligonucleotide primers, ranging in size from 37 to 56 nucleotides, were synthesized using a Model 8700 DNA synthesizer (Milligen/Bioresearch, Burlington, VT). They were purified on oligo-Pak columns (Milligen/Bioresearch) according to the supplier's recommendation. The sequences of the mutagenic primers were as follows, where the mutagenic changes are underlined:

V<sub>L</sub> CDR3:

5'-GCC AGC GCC GAA GCT GAG GGG ATA GCT ATA ATA CTG CTG ACA-  
3'

5'-GGT GCC AGC GCC GAA GCT GAG GGG GGT GCT ATA ATA CTG CTG  
5 ACA-3'

5'-GCC ACG GCC GAA TGT GTA GGG ATA GCT ATA ATA CTG CTG ACA  
-3'

5'-GCC GAA TGT GAG GGG GGT GCT ATA ATA CTG CTG ACA ATA-3'

10 V<sub>H</sub> CDR1:

5'-GTT TCA CCC AGT GCA TTG CAT AAT CAG TGA AGG TGT A-3'

V<sub>H</sub> CDR2:

5'-GTG GCC TTG CCC TGG AAC TTC TGT GAG TAC TTA AAA TCA TCG  
15 TTT CCG GGA GAG AA-3'

#### Example VIII. Assembly of PCR Products

The PCR products were assembled and sequenced as described in Example  
20 II. The 425 base pair (bp) PCR product obtained using the HuCC49 light chain  
construct as a template carried sequences encoding the leader peptide, the CC49 V<sub>L</sub>  
domain and the amino terminus of the kappa (k) constant region, terminating in a  
*Sac*II site located 10 bp downstream of the V<sub>L</sub>. Similarly, the 432 base pair (bp)  
PCR product from the heavy chain template encompassed sequences encoding the  
25 leader, the V<sub>H</sub> and the amino terminus of the C<sub>H</sub>1 domain, extending to the *Apal* site,  
which is located 17 bp downstream from the start of the C<sub>H</sub>1 domain.

#### Generation of Recombinant SDR Substituted CC49 MAbs

SDR substituted variants were generated essentially as described Example  
30 III, except for the following. The Sf900-II medium included 50 µg/ml of antibiotic,  
gentamicin and the infectious supernatants were harvested six days after  
transfection.

Purification of SDR Substituted CC49 Mab

Three days after infection, the tissue culture supernatant was harvested and clarified by centrifugation at 2000xg for 10 minutes. Tris buffer was added to the supernatant to a final concentration of 20 mM. Following incubation at 4°C for 2–3 hours, any contaminating proteins were pelleted by centrifugation at 10,000xg for 15 minutes. The supernatant was applied to a protein G agarose column (Gibco BRL) and the bound protein was eluted from the column, using 0.1 M glycine hydrochloride, pH 2.5. The pH of the eluted material was immediately adjusted to 7.0 with 1.0 M Tris buffer, pH 8.0. The protein was concentrated using a Centriplus 30 microconcentrator (Amicon, Beverly, MA), centrifuged at 3000xg for 80 minutes. The concentrated protein was recovered in phosphate-buffered saline (PBS). The protein concentration was determined by the as described in Example III. The purity of the antibody preparation was evaluated by electrophoresis on 4–12% SDS-PAGE, under reducing and non-reducing conditions. The proteins were visualized by staining with Coomassie blue, as described in Example III.

Example IX. Competition Radioimmunoassays for SDR Substituted VariantsELISA

The ability of the variants to express immunoglobulin molecules and their antigen reactivity of the heavy ( $^{32,34}\text{H}$  and  $^{60-62,64}\text{H}$ ) or variant light ( $^{94}\text{L}$ ,  $^{96}\text{L}$ ,  $^{97}\text{L}$ ,  $^{94,97}\text{L}$ ,  $^{97}\text{L}_{1,2}$  and  $^{94,97}\text{L}_{1,2}$ ) chain variants was evaluated using ELISA assays.

ELISA assays were carried out by coating individual wells of a 96-well polyvinyl microtiter plates with 1  $\mu\text{g}$ /well of TAG-72 positive bovine submaxillary mucin (BSM) (Sigma Chem. Co., St. Louis, MO), and following the procedure described by Bei et al., (1995) J. Immunol. Methods, 186:245–255.

Not all variant antibodies were positive for antigen binding activity. Results of the ELISA assay for the binding activity to the TAG-72 positive BSM showed that the variant antibodies specified by expression constructs carrying the variant genes  $^{32,34}\text{H}$  and  $^{96}\text{L}$  were not reactive with BSM. In contrast, variant antibodies expressed by  $^{97}\text{L}$  and  $^{60-62,64}\text{H}$  constructs showed strong BSM binding activity. While immunoglobulin molecules expressed by  $^{94}\text{L}$  and  $^{94}\text{L}_{1,2}$  constructs showed moderate positive antigen binding reactivity, those expressed by  $^{94,97}\text{L}_{1,2}$  were only weakly positive. (Figure 13)

A partial or complete loss of antigen binding activity of the variant immunoglobulins might be attributed to the detrimental effect of the SDR substitutions on the combining site of HuCC49. Alternatively, the plaques may show lower or no antigen binding reactivity because some of the expression constructs failed to express, were expressing at significantly lower level, or producing antibodies that were not physically normal. To examine these possibilities, variant antibodies were produced and purified from a larger batch of cells that were freshly infected with inoculum derived from the highest producing clone for each of the constructs. The concentration of the secreted variant antibodies in culture supernatants ranged between 2–3 µg/ml. Purified immunoglobulin molecules were characterized by SDS–PAGE. Under reducing conditions, immunoglobulin molecules expressed by each of the constructs yielded two bands that co-migrated with the heavy and light chains of HuCC49 MAb (data not shown). Antibodies produced by the insect cells harboring expression constructs <sup>97</sup>L<sub>1,2</sub> and <sup>94,97</sup>L<sub>1,2</sub> genes paired with the HuCC49 heavy chain gene showed similar results (data not shown). These results make it evident that all constructs expressed and produced comparable levels of immunoglobulin molecules of appropriate size. Therefore, it can safely be concluded that the variant HuCC49 MABs carrying <sup>96</sup>L and <sup>32,34</sup>H substitutions suffered a total loss of antigen binding activity.

#### Competition Radioimmunoassay

Competition radioimmunoassays (RIAs) were performed to determine relative binding of the variant MABs and the parental HuCC49 to BSM. Details of the procedure are described by Kashmiri et al., (1995) *Hybridoma*, 14:461–473. Serial dilutions of the purified unlabeled variant antibodies or the parental HuCC49 MAB were used to compete with radiolabeled HuCC49 for binding to the TAG–72 positive BSM. Briefly, 25 µl of serial dilutions of the purified SDR substituted variants or the parental HuCC49 in PBS containing 1% BSA were added to wells of 96–well microtiter plates containing 10 ng of BSM. 25 µl of <sup>125</sup>I–labeled HuCC49 (50,000 cpm) was added to each well to compete with the unlabeled parental or variant HuCC49 for binding to the BSM coated on the plates. The plates were incubated overnight at 4°C and then washed and counted in a γ–scintillation counter.

Competition profiles of the light chain variants presented in panel A show that the variant <sup>96</sup>L failed to compete, while all other variants antibodies competed with the parental HuCC49 completely and with similar slopes. (Figure 13)

However, the competition curves of all variants with the exception of  $^{97}\text{L}$  were shifted significantly to the right, indicating a loss of reactivity with antigen (BSM). This shift was notably less pronounced for  $^{97}\text{L}_{1,2}$ . Similarly, it is evident from the competition profiles of the heavy chain variants (panel B) that the variant MAb  $^{32,34}\text{H}$ , with substitutions in H-CDR1, did not inhibit binding of HuCC49 MAb to BSM, whereas  $^{60-62,64}\text{H}$ , the variant with substitutions in the H-CDR2, competed completely with a profile that was almost identical to that of the parental HuCC49.

The relative affinity constants were calculated as described in Example IV. The relative affinity constants ( $K_a$ ) of the variants were calculated from the linear parts of the competition curves. The  $K_a$  of  $^{97}\text{L}$  and  $^{60-62,64}\text{H}$  MAbs were  $3.6 \times 10^8 \text{ M}^{-1}$  and  $2.2 \times 10^8 \text{ M}^{-1}$ , respectively. These values are comparable to  $3.2 \times 10^8 \text{ M}^{-1}$ , the  $K_a$  of the parental HuCC49. The variant  $^{97}\text{L}_{1,2}$  was found to have a  $K_a$  of  $1.4 \times 10^8 \text{ M}^{-1}$ , which is approximately 2- to 3-fold less than the  $K_a$  of HuCC49 MAb.

Two new expression constructs were then generated and expressed in Sf9 cells; in one of them, the gene encoding the variant heavy chain  $^{60-62,64}\text{H}$  was paired with the gene encoding the light chain variant  $^{97}\text{L}$ . Gene  $^{60-62,64}\text{H}$  was paired with the  $^{97}\text{L}_{1,2}$  light chain gene in the other construct. Competition profiles of the purified antibodies show that these variant MAbs competed completely with HuCC49 MAb for antigen binding, yielding competition curves of the same slope as HuCC49. (Figure 13) The relative affinity constant of the Variant MAb  $^{97}\text{L}/^{60-62,64}\text{H}$  was  $5.48 \times 10^8 \text{ M}^{-1}$ , a figure favorably comparable to that of HuCC49, while the  $K_a$  of the variant MAb  $^{97}\text{L}_{1,2}/^{60-62,64}\text{H}$  was  $1.15 \times 10^8 \text{ M}^{-1}$ , which is about 3-fold less than that of the parental HuCC49 MAb.

#### 25 Example X. High Performance Liquid Chromatography

In a reported Phase I clinical trial, in which  $^{177}\text{Lu}$ -labeled MAb CC49 was administered to adenocarcinoma patients, several patients were found to have anti-idiotypic antibodies to MAb CC49. Sera collected from the study was used to examine the potential immunogenicity of the variants. The sera was obtained by separating the blood by centrifugation. High Performance Liquid Chromatography (HPLC) was used to determine antigen reactivity of the variants by monitoring complex formation between antibodies in the patient sera and the variant MAbs.

Prior to HPLC analysis, any free TAG-72 and human anti-murine antibodies other than anti-idiotypic antibodies to CC49 present in the sera were absorbed out



using MAb CC92 conjugated to a solid support. MAb CC92 is a murine anti-TAG-72 antibody which has the same isotype as CC49 and recognizes an epitope of TAG-72 other than that recognized by CC49. Patient sera was then incubated with  $^{125}\text{I}$ -labeled HuCC49 (approximately 500,000 cpm) and 5  $\mu\text{g}$  of the cold competitor; either HuCC49 or one of the variant MAbs.

The competition assay is described in Example V. Briefly, patient sera was mixed with  $\sim 0.3 \mu\text{Ci}$  of  $^{125}\text{I}$ -HuCC49 and serial dilutions of purified HuCC49 or its variants. Prior to the assay, the amount of sera required in half-maximal immune complex formation was determined. The mixture was brought to a final volume of 50  $\mu\text{l}$ . 25  $\mu\text{l}$  of the final solution was applied to a 7.8 mm x 30 cm TSK3000 analytical column (Tosohaas, Montgomeryville, PA) and eluted at 0.5 ml/min with elution buffer (100 mM KCl in 67 mM sodium phosphate, pH 6.8). Radioactivity was monitored using a flow-through Model 170  $\gamma$ -scintillation detector (Beckman).

Complex formation of the radiolabeled HuCC49 with the anti-idiotypic antibodies in patient sera reduced the retention time of the radiolabel on the column. The ability of the variant to inhibit complex formation with  $^{125}\text{I}$ -labeled HuCC49 was determined by the differential in the retention time of the radiolabel on HPLC column, when a mixture of sera and  $^{125}\text{I}$ -labeled HuCC49 was loaded on the column with or without incubation with the cold competitor. Inhibition of complex formation by a competitor indicates that the competitor shares the immunogenic epitope with HuCC49. (Figure 14)

From an analysis of the percent of input counts recovered as a complex, when a mixture of  $^{125}\text{I}$ -labeled HuCC49 and sera from each of the four patients was incubated with 5  $\mu\text{g}$  of cold competitor and subjected to HPLC analysis, it is evident that the variant antibodies  $^{97}\text{L}$  and  $^{32,34}\text{H}$ , like HuCC49, inhibited complex formation. In contrast, the variant MAbs  $^{96}\text{L}$  and  $^{94,97}\text{L}_{1,2}$ , like the nonspecific Human immunoglobulin did not inhibit complex formation of HuCC49 with sera from any patient except EA. Complex formation with EA sera was partially inhibited by the two variants. The variant MAbs  $^{94}\text{L}$ ,  $^{94,97}\text{L}$ ,  $^{97}\text{L}_{1,2}$  and  $^{60-62,64}\text{H}$  inhibited complex formation only partially with sera from all patients. The variant  $^{97}\text{L}^{60-62,64}\text{H}$ , whose antigen binding activity was comparable to that of parental HuCC49, inhibited sera of three patients (DG, CP and DS) only partially, but completely inhibited the sera from EA patient to form complexes with HuCC49. More importantly, the variant  $^{97}\text{L}_{1,2}^{60-62,64}\text{H}$  did not compete with HuCC49 to form complex with anti-idiotypic antibodies present in sera from two patients (CP and

DS) while showing only partial competition with sera from two other patients (DG and DS).

Using serial dilutions of the competitors, competition profiles were developed to determine the relative amounts of unlabeled competitor antibodies required to achieve 50% competition of the binding of  $^{125}\text{I}$ -labeled HuCC49 to the anti-idiotypic antibodies present in sera from one of the patients (CP). The percent inhibition of complex formation was calculated and plotted versus the concentration of competitor.

The competition profiles show that the cold HuCC49 competed completely and it required approximately 250 ng of the parental HuCC49 antibody to achieve 50% competition. In contrast, variant  $^{97}\text{L}_{1,2}/^{60-62,64}\text{H}$  inhibited binding of the radiolabeled HuCC49 to the sera anti-idiotypic antibodies only minimally; even 1  $\mu\text{g}$  of the variant failed to achieve more than 25% competition, that was achieved by 60 ng of HuCC49. This variant, which retains moderate antigen binding activity and reacts with patient's sera only minimally, might be most advantageous for clinical applications. This variant was further studied for plasma clearance and biodistribution in an animal model.

Figure 16 is a graph showing the immunoreactivity of variant  $^{97}\text{L}_{1,2}/^{60-62,64}\text{H}$  to human sera containing anti-murine CC49 variable region antibodies as assessed by HPLC analysis. The percent inhibition of the complex formation was calculated and plotted versus ng of the competitors. The competitors were HuCC49 (■) and variant (□).

#### Example XI. Biodistribution and Pharmacokinetic Studies

##### Pharmacokinetics

Since the rate of plasma clearance has a bearing on *in vivo* tumor targeting, a comparison of the pharmacokinetics of the variant to the parental HuCC49 was assessed using the procedures described by Kashmiri et al., (1995) Hybridoma, 14:461-473.

To study pharmacokinetics, athymic mice bearing TAG-72 positive LS-174T tumors (Colcher et al., (1983) Cancer Res., 43:736-742) were injected intravenously in the tail vein with a mixture containing 1.4  $\mu\text{Ci}$   $^{131}\text{I}$ -labeled HuCC49 and 4.4  $\mu\text{Ci}$   $^{125}\text{I}$ -labeled variant MAb  $^{97}\text{L}_{1,2}/^{60-62,64}\text{H}$ . Blood samples were collected at various time points via the tail vein into 10  $\mu\text{l}$  heparinized capillary tubes

(Drummond, Broomall, PA). The amounts of  $^{131}\text{I}$  and  $^{125}\text{I}$  in the plasma were determined and corrected for the respective rates of the decay of the two radionuclides. The percentage of the injected dose of each radionuclide remaining in the plasma was then calculated for each time point. The results suggest that the blood clearance patterns of the two antibodies are not significantly different. (Figure 17). For 50% of the injected dose of the HuCC49 or variant to clear the blood compartment, required 1 and 2 hours, respectively. At 24 hours, 85% and 80% of the radiolabeled HuCC49 and the variant, respectively, was cleared from the blood. At 48 hours, the percentage of HuCC49 and the variant cleared from the blood was 92% and 88%, respectively.

#### Biodistribution

Biodistribution assays were performed as described by Kashmiri et al., (1995) Hybridoma, 14:461-473. To investigate the ability of the variant HuCC49 MAb to localize to human tumor xenograft and determine radiolocalization index (RI), athymic mice bearing TAG-72 positive LS-174T tumors (Colcher et al., (1983) Cancer Res., 43:736-742) were injected intravenously in the tail vein with a mixture containing 1.4  $\mu\text{Ci}$   $^{131}\text{I}$ -labeled HuCC49 and 4.4  $\mu\text{Ci}$   $^{125}\text{I}$ -labeled variant MAb  $^{97}\text{L}_{1,2}/^{60-62,64}\text{H}$ . The amount of  $^{131}\text{I}$  and  $^{125}\text{I}$  were determined in blood samples collected via tail vein at specified times. For each time point, 5 mice were sacrificed to collect and weigh tumor, blood and all other major organs. Radioactivity was measured in a  $\gamma$ -scintillation counter and it was corrected for the decay. The percentage of the injected dose per gram (%ID/gm) for each organ was determined.

The % injected dose of the two antibodies per gram of either tumor or different normal tissues that were collected at different time points shows that the biodistribution patterns of the two antibodies are essentially the same. Both showed significant tumor localization by 24 hours. (Figure 18) By 48 hours, when only 8% and 12% of the injected dose was present in the blood, 17.6% and 23.8% ID/b of HuCC49 and the variant were, respectively, present in the tumor.

#### Example XII. Characterization of Humoral Immune Response against CC49

In this Example, the humoral immune response against HuCC49 CDR-replacement variants is examined.

Generation of Humanized CC49 (HuCC49) and Humanized CC49 CDR-  
Replacement Variants (CDR variants)

A clone producing humanized CC49 (HuCC49) was grown in protein free  
5 hybridoma medium PFHM-II (GIBCO-BRL, Gaithersburg, MD) as described by  
Kashmiri (1995), Hybridoma, 14:461-473. The humanized CC49 monoclonal  
antibody (MAb) was purified from the tissue culture supernatant by Protein G  
affinity chromatography as described by Kashmiri (1995), Hybridoma, 14:461-473.

Seven HuCC49 CDR-variants were produced as described in Examples I-  
10 III.

Radiolabeling

MAb HuCC49, BL-3 and the CDR-replacement variants of HuCC49 were  
labeled with Na<sup>125</sup>I using the iodogen method (Pierce, Rockford, IL) as described by  
15 Fraker et al. (1978), Biochem. Biophys. Res. Commun. 80:849-857; and Colcher et  
al. (1988), Cancer Res., 48:4597-4603. BL-3 is an isotype-matched control for  
CC49 (described by Colcher et al. (1987), Cancer Res., 47:4218-4224). The  
labeling procedure typically resulted in specific activities of 5-10  $\mu$ Ci/ $\mu$ g.

20 Patients and Sample Collection

Patients with recurrent metastatic adenocarcinoma were enrolled in a Phase I  
Study to assess the maximum tolerated does of intravenously administered  
<sup>177</sup>Lutetium radiolabeled MAb CC49 (Mulligan, (1995) Clin. Cancer Res. 1:1447-  
1454).

25 In the Phase I Study, adenocarcinoma patients were given a test dose of 0.1  
mg (i.v. bolus) of MAb CC49 and observed for 30 minutes prior to administration of  
the <sup>177</sup>Lu-labeled MAb CC49. The radiolabeled MAb was given as a 1 hour i.v.  
infusion. Blood samples were collected prior to and at the end of the infusion, and  
0.5, 1 and 2 hours after the infusion, and afterward, daily for 7 days. Patients  
30 returned for a follow-up examination at 3, 6 or 8 weeks, at which time blood  
samples were collected. Sera was separated and stored at -20°C until analyzed.  
Sera from these patients provided a resource for assessing the humoral response of  
patients to the murine MAb CC49. The patient characteristics are presented in Table  
1, below.

**Table 1: Patient Characteristics**

Dose Level	Patient	Age	Sex	Tumor	Dose <sup>a</sup>	
					mCi	mg MAb
10 mCi/m <sup>2</sup>	DS	52	F	Breast	16.0	20
	LW	45	F	Breast	19.0	20
	JJ	61	F	Breast	17.2	20
25 mCi/m <sup>2</sup>	DG	45	F	Breast	41.0	20
	LJ	45	F	Breast	40.3	20
	JM	42	F	Breast	45.4	20
15 mCi/m <sup>2</sup>	JG	61	M	Colon	29.8	44
	RW	46	F	Lung	24.2	20
	TD	50	M	Colon	31.5	47
	EA <sup>b</sup>	53	F	Colon	24.2	20
	CP <sup>b</sup>	53	F	Colon	26.0	20
	LQ <sup>b</sup>	45	F	Colon	29.7	20

<sup>a</sup> Patients were administered <sup>177</sup>Lu-PA-DOTA-CC49 by intravenous injection.

<sup>b</sup> Patient received new formulation of <sup>177</sup>Lu-PA-DOTA-CC49 that was labeled using a modification of the method described by Mulligan et al. (1995), *Clin. Cancer Res.* 1:1447-1454.

PA-DOTA was conjugated to human serum albumin (HSA), radiolabeled with Na<sup>125</sup>I, incubated with the patient sera and analyzed for immune complex formation by size-exclusion HPLC. None of the sera showed detectable reactivity with the PA-DOTA-HSA conjugate (Data not shown).

#### Determination of Patient Humoral Response

The sera from the twelve patients was evaluated for the presence of human anti-murine antibodies (HAMA) in response to MAb CC49 using high performance liquid chromatograph (HPLC) as described by Mulligan et al. (1996) *Clin. Cancer Res.*, 1:1447-1454. The analysis was performed by adding about 500,000 cpm (0.4μCi) of <sup>125</sup>I-BL-3 to 50 μl of patient sera. Following a 60 minute incubation at 37°C, 25 μl of the mixture was applied to a size-exclusion column (TSK 3000SW; TosoHaas, Montgomeryville, PA) equilibrated in 67 mM sodium phosphate (pH 6.8) containing 100 mM KCl. The sera samples were eluted at a flow rate of 0.5 ml/min. The protein was detected by absorbance at 280 nm and the radioactivity was measured using a flow-through γ-scintillation counter (Model 170, Beckman Instruments, Inc., Berkeley, CA). The presence of HAMA was indicated by a shift in the elution profile of the <sup>125</sup>I-BL-3 because the formation of immune complexes with the radiolabeled BL-3 results in a shorter retention time. The patients' pre-study sera, normal human sera and phosphate buffered saline with <sup>125</sup>I-BL-3 were used as controls. A patient with a known HAMA response from a previous study

(Colcher et al. (1990), *J. Nucl. Med.*, 31:1133-1142) served as a positive control. The patients' sera were demonstrated to have antibodies against the variable region of the murine CC49.

Figure 19 shows an HPLC analysis of patient HAMA following intravenous injection of  $^{177}\text{Lu}$ -CC49. Serum samples from LQ were analyzed for the presence of HAMA at various timepoints before and after injection with 20 mg of  $^{177}\text{Lu}$ -labeled CC49. Pre-study sera (A), sera collected at 7 days (B), 3 weeks (C), and 6 weeks (D) were mixed with  $^{125}\text{I}$ -BL-3 and applied to a size exclusion column. Reduction in retention time of the radiolabeled BL-3 as compared to migration of the  $^{125}\text{I}$ -BL-3 in buffer (E) were indicative of immune complex formation and therefore the presence of HAMA.

Lack of complex formation is evident (FIG. 19A) when the pre-study sera of Patient LQ is incubated with the  $^{125}\text{I}$ -BL-3. All of the radioactivity is associated with the peak at about 18.5 minutes, the same retention time for  $^{125}\text{I}$ -BL-3 in buffer (FIG. 19E). Complex formation is also absent when the sera collected at seven days is incubated with  $^{125}\text{I}$ -BL-3 (FIG. 19B). With sera collected at 3 weeks (FIG. 19C), however, there is an indication of complex formation (46%) with the appearance of two peaks with a shorter retention time (i.e., 14 and 16 minutes). The peaks at a shorter retention time indicate the development of a higher molecular weight species in the sera. At 6 weeks (FIG. 19D), the HAMA response has increased, the amount of radioactivity bound in complexes is now 66%.

Figure 20 shows an HPLC analysis of patients' humoral response to the variable region of MAb CC49. The percent complex formation has been plotted versus time for (solid lines) patients DS (O), LW ( $\square$ ), JJ ( $\Delta$ ), DG ( $\bullet$ ), LJ ( $\blacksquare$ ), TD ( $\blacktriangle$ ); (dotted lines) JG (O), RW ( $\square$ ), JM ( $\Delta$ ), EA ( $\bullet$ ), CP ( $\blacksquare$ ), LQ ( $\blacktriangle$ );

At one week, none of the patients showed a detectable response against the HuCC49 (FIG. 20). At 3 weeks, sera from nine of the twelve patients (75%) appears to contain antibody against the variable region of CC49 with one patient having a notably higher response than the others. For the eleven patients evaluated at six weeks, only two patients did not elicit a human antiviral region antibody response (HAVRA) against CC49, i.e., 9 of 11 evaluable patients (82%) had antibody against the variable region of the murine MAb CC49.

Three patterns of HAMA-HAVRA response are evident. The patterns of the HAMA and HAVRA responses elicited in each of the patients were very similar, differing only in the apparent level of antibody. Patients DG, LW, LQ and CP

developed HAVRA simultaneously with HAMA. Patients DS and JM appear to have a strong HAVRA, while HAMA response is modest. While in patients TD, JG, and EA, the HAVRA level is lower than HAMA at 3 weeks, followed by HAMA and HAVRA attaining high levels at later timepoints. In no patient was there a

5 HAVRA response without the development of HAMA..

The HAMA results for the twelve patients are summarized below in Table 2.

**Table 2: HPLC Analysis of Patients' Anti-mouse immunoglobulin response after i.v. injection of  $^{177}\text{Lu}$ -CC49**

Patient	Days Post-Injection of $^{177}\text{Lu}$ -CC49			
	7	21	42	56
DS	0 <sup>a</sup>	1	16	27
LW	3	6	81	NA
JJ	0	12	3	4
DG	0	24	84	NA
LJ	0	42	NA	NA
JM	0	8	47	NA
JG	4	83	83	NA
RW	0	1	2	NA
TD	0	95	100	NA
EA	0	27	100	100
CP3	0	33	27	NA
LQ	0	46	66	100

<sup>a</sup> The values are the percent of  $^{125}\text{I}$ -BL-3 detected in complexes after a brief incubation with the patient sera and resolved by size-exclusion chromatography. The timepoints of each patient are background corrected using the patients' pre-study sera.

The patterns of the HAMA responses are varied and are consistent with previous findings by Colcher et al. (1990), J. Nucl. Med. 31:1133-1142. Ten out of the twelve patients (83%) demonstrate a HAMA response at 3 weeks following a single intravenous injection of 20 mg  $^{177}\text{Lu}$ -labeled CC49, two patients (LW and JG) have minimal responses evident at 7 days with complexes of 3% and 4%, respectively. One patient (RW) may be considered a nonresponder. Some of the patients show an escalating HAMA response, while others plateau. Yet another (JJ) peaks at 3 weeks, followed by an apparent decrease in the HAMA level. Overall, at 3 weeks, 8 of 12 patients (57%) at and 6 weeks, 9 of 11 (82%) were HAMA positive.

#### Specificity of Patient Response

The specificity of the patients' antibody response to CC49 was assessed using  $^{125}\text{I}$ -labeled HuCC49 and HuCC49 CDR-replacement variants to determine whether or not any of the responses were directed against the variable region of CC49. To accomplish this, the HPLC methodology was employed using  $^{125}\text{I}$ -HuCC49 as the probe (See, Kashmiri et al. (1995), Hybridoma, 14:461-473).

To eliminate the artifactual influence of TAG-72 in the HPLC analysis for anti-CC49 antibody responses found in the patient's serum, immunoadsorbents were prepared as reported by Ferroni et al. (1992) J. Clin. Lab. Analysis, 4:465-473. For the purpose of these studies, purified MAb CC92 was coupled to Reacti-gel (HW65F, Pierce) according to the method of Heam et al. (1979), J. Chromatog., 185:463-470. MAb CC92 is a second-generation monoclonal antibody that reacts with TAG-72, but with an epitope distinct from the one recognized by CC49.

Before probing the patients' sera with the  $^{125}\text{I}$ -HuCC49, removal of HAMA and circulating TAG-72 were confirmed using  $^{125}\text{I}$ -BL-3 and  $^{125}\text{I}$ -B72.3, respectively (data not shown). MAb B72.3 is an anti-TAG-72 MAb that has been shown to form complexes with TAG-72 in patient sera (Colcher et al. (1990), J. Nucl. Med., 31:1133-1142).

In the competition assay, 5  $\mu\text{g}$  of the cold competitor (either purified HuCC49 or one of its variants) was added to a mixture of patient sera (collected 8 weeks post-i.v. injection with  $^{177}\text{Lu}$ -CC49) and  $^{125}\text{I}$ -HuCC49 and then analyzed by size-exclusion chromatography for the absence or presence of complexes. The percent inhibition of complex formation was calculated. If the variant competed with the  $^{125}\text{I}$ -labeled MAb, and complex formation was inhibited, then the variant



still contained the immunodominant CDR. If the variant failed to inhibit complex formation, then the CDR that is no longer present in the variant is recognized by the patient and hence it is an immunogenic CDR. An example of this assay (using serum from patient LQ) is shown in FIG. 21. Panel A is the profile of the <sup>125</sup>I-HuCC49 in buffer only. Panel B, is the profile showing complex formation (42.9%) resulting from patient sera (LQ) incubated with <sup>125</sup>I-HuCC49. When HuCC49 is added as a competitor, there is competition for the <sup>125</sup>I-HuCC49 and a loss or absence of complexes is observed (Panel C). The same is true of a variant which still contains an immunogenic CDR (e.g., light chain CDR2 as the competitor) (Panel D). In contrast, there is either a partial (Panel F) or total retention of the complexes (Panel E), when light chain CDR1 or CDR3 variants, respectively, are the competitors.

The results are very striking, see Table 3.

**Table 3: HPLC Analysis of Patient Reactivity to CDR-Replacement variants of HuCC49<sup>a</sup>**

Competitor		Patient					
	CDR <sup>b</sup>	DS	DG	JG	EA	CP	LQ
None	—	33.5 <sup>c</sup>	46.2	24.5	56.8	32.2	42.9
HuCC49	—	0	0	2.6	0.5	1.5	3.0
Hu IgG	—	46.4	59.0	25.1	63.6	ND	54.1
Light Chain	1	16.0	12.2	9.8	10.1	16.9	14.3
	2	2.7	3.4	2.7	4.4	3.0	2.4
	3	34.8	48.2	22.4	37.6	33.5	46.7
	1,2	24.6	24.5	12.6	19.4	15.7	20.2
Heavy Chain	1	10.2	3.9	3.3	7.0	5.8	3.5
	2	32.7	32.5	12.7	24.7	29.7	36.6
	3	7.3	5.1	3.7	8.2	6.7	4.6

<sup>a</sup> The sera from patients injected with <sup>177</sup>Lu-CC49 were tested for reactivity with variants of HuCC49 in which individual CDRs had been substituted with human sequences in both the heavy and light chains of HuCC49. Five µg of the purified CDR-replacement variants were added to a mixture of <sup>125</sup>I-HuCC49 and the patient sera and then analyzed for the presence or absence of immune complex formation.

<sup>b</sup> The number indicates which CDR in the HuCC49 has been replaced with a human CDR sequence.

<sup>c</sup> The values are the percent of complexes, the higher molecular weight species, resolved by size-exclusion chromatography.

Of the six patients analyzed, all six demonstrated reactivity with CDR3 light chain indicating that light chain CDR3 may be immunodominant in murine CC49 MAb. In the heavy chain, CDR2 appears to be dominant but not with the same level of consensus (four of the six patients show the same level of reactivity, the other two

demonstrated partial reactivity). Concordance was obtained among the six patients in regard to CDR2 of the light chain and CDR1 and CDR3 of the heavy chain, which do not appear to contribute to the immunogenicity of the MAb. This is also the case with the light chain CDR1 and, it follows, the variant with the dual substitution of CDR1 and 2 in the light chain, in which all six patients displayed a partial recognition of the variants. Partial recognition with the heavy chain CDR2 variant with two patients may be due to a loss of part but not all of the cognizant epitope, a change in the conformation or conformational epitope, or loss of amino acid residues that might stabilize the antibody:antibody interaction.

#### Quantitation of Patient Antibody Response

Quantitation of the HAMA or anti-variable region antibody levels in four patients was performed using HPLC analysis. The quantitation study was performed by adding either 500 ng of unlabeled BL-3 or 250 ng of HuCC49, respectively, to the mixture of patient serum and <sup>125</sup>I-HuCC49 and calculating the amount of BL-3 or HuCC49 bound in complexes.

As shown in Table 4, below, at 6 weeks, the amount of HAMA varies from patient to patient by 43-fold, while the variability of HAVRA is within 4-fold. Furthermore, the HAMA versus HAVRA levels may vary from 10 to 145-fold. Clearly, HAVRA can be detected at 3 weeks, and, not surprisingly, it does not appear to attain the same levels as HAMA. In patient EA, there is a dramatic 10-fold increase in the level of HAVRA from 6 to 8 weeks that is noteworthy.

**Table 4: Quantitation of anti-CC49 variable region and anti-murine response of patients administered <sup>177</sup>Lu-CC49**

Patient	<u>µg of Ab/ml Sera</u>		
	Post-Mab Injection	BL-3 <sup>a</sup>	HuCC49 <sup>b</sup>
EA	0	0	0
	3 weeks	4.1	0.3
	6 weeks	289.0	2.3
	8 weeks	314.4	21.6
CP	0	0	0
	3 weeks	16.0	0.8
	5 weeks	25.2	0.7
	6 weeks	23.2	0.7
LQ	0	0	ND
	3 weeks	4.61	0.4
	6 weeks	6.64	0.7
	8 weeks	ND	1.7
JG	0	0	0
	3 weeks	58.6	0.7
	6 weeks	47.8	2.6

Competition Radioimmunoassay

To confirm whether the HAVRA was actually an anti-idiotypic response, including internal image anti-idiotypic antibodies, to the murine MAb CC49, the sera from one patient (EA) was selected and assessed for blocking of the binding of  $^{125}\text{I}$ -HuCC49 to BSM in a radioimmunoassay.

The immunoreactivity of the radiolabeled MAbs was assessed using bovine submaxillary mucin (BSM) immobilized on a solid support (Reacti-Gel HW65, Pierce) as a modification of the method reported by Heam et al. (1979), J. Chromatog., 185:463-470 and Schott (1992) Cancer Res., 52:6413-6417. Briefly, bovine submaxillary mucin (BSM), which is TAG-72 positive, was adsorbed to each well of a 96-well polyvinylchloride microtiter plate at 10 ng in 50 $\mu\text{l}$  of phosphate buffered saline (pH 7.2) as described by Horan Hand et al. (1992), Cancer Immunol. Immunother., 353:165-174. After treating the wells with 5% BSA in PBS, serial dilutions of the patient sera (25 $\mu\text{l}$  in 1% BSA in PBS) were added to each;  $^{125}\text{I}$ -CC49 (38 nCi in 25  $\mu\text{l}$ ) was also added. Following an 18 hour incubation at 4°C, the plates were washed and the wells counted in a  $\gamma$ -scintillation counter. The percent inhibition was calculated and compared to that of unlabeled CC49. Human IgG (Organon Teknika, Durham, NC), which does not react with TAG-72 was included as a control antibody.

It was found that the patient sera could block the binding of  $^{125}\text{I}$ -HuCC49 with BSM (FIG. 22) suggesting that the patient, in actuality, demonstrates an anti-idiotypic response, consisting of the internal image anti-idiotypic antibodies. Furthermore, the anti-idiotypic response was observed to increase over an eight week period. Figure 22 shows the detection of patient (EA) anti-idiotypic antibody response to murine CC49: pre-study sera from patient EA ( $\square$ ); sera collected at 3 weeks (A), 6 weeks (B), and 8 weeks (C).

All references cited in this disclosure are hereby incorporated by reference.

**WHAT IS CLAIMED IS:**

1. A humanized anti-TAG-72 antibody comprising:  
light chain Complementarity Determining Regions (L-CDRs), comprising L-CDR1, L-CDR2 and L-CDR3; and heavy chain Complementarity Determining Regions (H-CDRs), comprising H-CDR1, H-CDR2 and H-CDR3,  
wherein L-CDR3, H-CDR1, H-CDR2 and H-CDR3 are from a non-human antibody and at least one of L-CDR1 and L-CDR2 are human antibody sequences.
2. The humanized antibody of claim 1, wherein L-CDR1 is from a human antibody.
3. The humanized antibody of claim 2, wherein L-CDR1 is from human monoclonal antibody LEN.
4. The humanized antibody of claim 1, wherein L-CDR2 from a human antibody.
5. The humanized antibody of claim 4, wherein L-CDR2 is from human monoclonal antibody LEN.
6. The humanized antibody of claim 1, wherein both L-CDR1 and L-CDR2 are human antibody sequences.
7. The humanized antibody of claim 1, wherein L-CDR1 and L-CDR2 are human antibody sequences from the same human antibody.
8. The humanized antibody of claim 7, wherein L-CDR1 and L-CDR2 are human antibody sequences from human monoclonal antibody LEN.
9. The humanized antibody of claim 6, wherein L-CDR1 and L-CDR2 are human antibody sequences from different human antibodies.

10. The humanized antibody of claim 1, wherein L-CDR3, H-CDR1, H-CDR2 and H-CDR3 are from murine monoclonal antibody CC49.
11. A humanized anti-TAG-72 antibody comprising:  
light chain Complementarity Determining Regions (L-CDRs),  
comprising L-CDR1, L-CDR2 and L-CDR3; and heavy chain  
Complementarity Determining Regions (H-CDRs), comprising H-CDR1,  
H-CDR2 and H-CDR3,  
wherein at least one amino acid of positions 60, 61, 62, or 64 in H-  
CDR2 is replaced with a corresponding amino acid from a human antibody.
12. The humanized antibody of claim 11, wherein the human antibody is  
21/28'CL.
13. The humanized antibody of claim 11, wherein the amino acid at position 97  
of L-CDR3 is replaced with a corresponding amino acid from a human  
antibody.
14. The humanized antibody of claim 11, wherein at least one of L-CDR1 and  
L-CDR2 are human antibody sequences.
15. The humanized antibody of claim 14, wherein L-CDR1 is a human antibody  
sequence.
16. The humanized antibody of claim 15, wherein L-CDR1 is from human  
monoclonal antibody LEN.
17. The humanized antibody of claim 14, wherein L-CDR2 is a human antibody  
sequence.
18. The humanized antibody of claim 17, wherein L-CDR2 is from human  
monoclonal antibody LEN.

19. The humanized antibody of claim 17, wherein both L-CDR1 and L-CDR2 are human antibody sequences.
20. The humanized antibody of claim 19, wherein L-CDR1 and L-CDR2 are human antibody sequences from the same human antibody.
21. The humanized antibody of claim 20, wherein L-CDR1 and L-CDR2 are from human monoclonal antibody LEN.
22. The humanized antibody of claim 19, wherein L-CDR1 and L-CDR2 are human antibody sequences from different human antibodies.
23. A humanized anti-TAG-72 antibody comprising:
  - light chain Complementarity Determining Regions (L-CDRs), comprising L-CDR1, L-CDR2 and L-CDR3; and heavy chain Complementarity Determining Regions (H-CDRs), comprising H-CDR1, H-CDR2 and H-CDR3,
  - wherein an amino acid at position 97 of L-CDR3 is replaced with a corresponding amino acid from a human antibody.
24. The humanized antibody of claim 23, wherein at least one amino acid of positions 60, 61, 62, or 64 in H-CDR2 is replaced with a corresponding amino acid from a human antibody.
25. The humanized antibody of claim 23, wherein at least one of L-CDR1 and L-CDR2 are human antibody sequences.
26. The humanized antibody of claim 25, wherein L-CDR1 is a human antibody sequence.
27. The humanized antibody of claim 26, wherein L-CDR1 is from human monoclonal antibody LEN.

28. The humanized antibody of claim 25, wherein L-CDR2 is a human antibody sequence.
29. The humanized antibody of claim 28, wherein L-CDR2 is from human monoclonal antibody LEN.
30. The humanized antibody of claim 25, wherein both L-CDR1 and L-CDR2 are from human antibody sequences.
31. The humanized antibody of claim 30, wherein L-CDR1 and L-CDR2 are human antibody sequences from the same human antibody.
32. The humanized antibody of claim 31, wherein L-CDR1 and L-CDR2 are from human antibody sequences from human monoclonal antibody LEN.
33. The humanized antibody of claim 30, wherein L-CDR1 and L-CDR2 are human antibody sequences from different human antibodies.
34. A humanized anti-TAG-72 antibody comprising:
  - light chain Complementarity Determining Regions (L-CDRs), comprising L-CDR1, L-CDR2 and L-CDR3; and heavy chain Complementarity Determining Regions (H-CDRs), comprising H-CDR1, H-CDR2 and H-CDR3,
  - wherein residues at positions 94 and 97 in L-CDR3 are from a non-human anti-TAG-72 antibody.
35. A humanized anti-TAG-72 antibody comprising:
  - light chain Complementarity Determining Regions (L-CDRs), comprising L-CDR1, L-CDR2 and L-CDR3; and heavy chain Complementarity Determining Regions (H-CDRs), comprising H-CDR1, H-CDR2 and H-CDR3,
  - wherein residues at positions 31, 32 and 34 in H-CDR1 are from a non-human anti-TAG-72 antibody.





46. A method of detecting cancer cells, comprising:  
contacting cells with composition comprising a polypeptide capable of specifically binding TAG-72, said polypeptide comprising a functional fragment of the humanized antibody of any of claims 11, 11, 23, 34 or 35.
47. The method of claim 46, wherein the polypeptide comprises a fragment selected from the group consisting of Fv, Fab, and F(ab')<sub>2</sub>.

CORRECTED  
VERSION\*

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

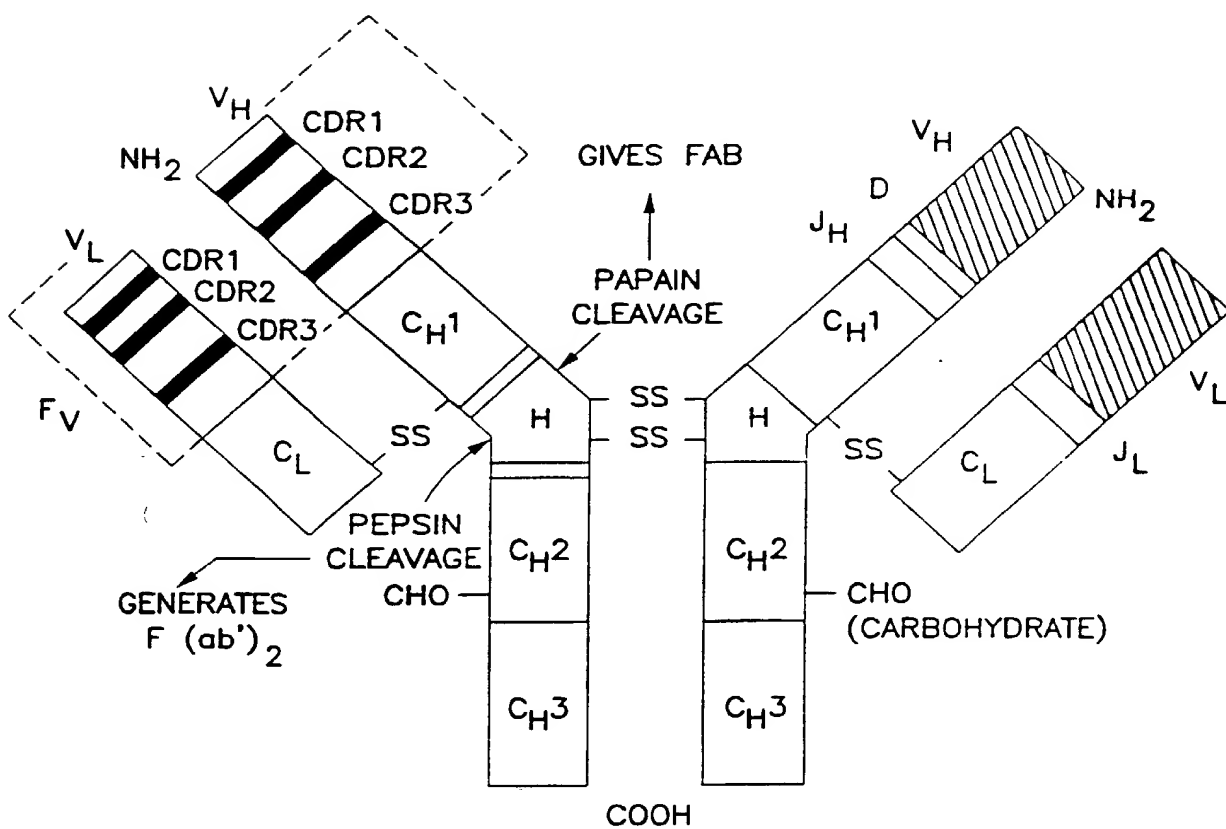


KMC

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification <sup>7</sup> : C12N 15/85, 15/62, 5/10, C07K 16/30, 16/46, A61K 51/10, A61P 35/00, G01N 33/574, A61K 39/395</p>	<p>A1</p>	<p>(11) International Publication Number: <b>WO 00/26394</b>  (43) International Publication Date: 11 May 2000 (11.05.00)</p>
<p>(21) International Application Number: PCT/US99/25552 (22) International Filing Date: 29 October 1999 (29.10.99) (30) Priority Data: 60/106,534 31 October 1998 (31.10.98) US 60/106,757 2 November 1998 (02.11.98) US (71) Applicant (for all designated States except US): THE GOVERNMENT OF THE UNITED STATES OF AMERICA as represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; Suite 325, 6011 Executive Boulevard, Rockville, MD 20852 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): KASHMIRI, Syed, V., S. [US/US]; 11541 Brandy Hall Lane, Gaithersburg, MD 20878 (US). PADLAN, Eduardo, A. [US/US]; 4006 Simms Drive, Kensington, MD 20895 (US). SCHLOM, Jeffrey [US/US]; 10301 Sorrel Avenue, Potomac, MD 20854 (US). (74) Agent: DAIGNAULT, Ronald, A.; Merchant &amp; Gould P.C., 3100 Norwest Center, 90 South Seventh Street, Minneapolis, MN 55402-4131 (US).</p>		<p>(81) Designated States: AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), DM, EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: VARIANTS OF HUMANIZED ANTI-CARCINOMA MONOCLONAL ANTIBODY CC49</p>		
<p>(57) Abstract</p> <p>The invention is directed towards mouse-human chimeric variants of CC49 monoclonal antibodies with minimal murine content. A first aspect of the invention provides CDR variants of humanized monoclonal antibody (HuCC49) in which less than all six (three heavy chain and three light chain) Complementarity Determining Regions (CDRs) of CC49 are present. A second aspect of the invention provides SDR variants of humanized monoclonal antibody (HuCC49) in which only Specificity Determining Regions (SDRs) of at least one CDR from CC49 are present. The invention is also directed towards biotechnological methods of making the variants and therapeutic methods of using the variants.</p>		

FIG. 1



WO 00/26394

FIG. 2

Light chain

CDR 1  
HuCC49  
LEN  
24 Lys 25 Ser 26 Ser 27 Gln 28 Ser 29 Gln 30 Lys 31 Asn 32 Tyr 33 Leu 34 Ala

CDR 2  
HuCC49  
LEN  
50 Trp 51 Ala 52 Ser 53 Ala 54 Arg 55 Glu 56 Ser

CDR 3  
HuCC49  
LEN  
89 Gln 90 Gln 91 Tyr 92 Tyr 93 Ser 94 Tyr 95 Pro 96 Leu 97 Thr

Heavy chain

CDR 1  
HuCC49  
21/28'CL  
31 Asp 32 His 33 Ala 34 Ile 35 His

CDR 2  
HuCC49  
21/28'CL  
50 Tyr 51 Phe 52 Ser 53 Gly 54 Asn 55 Asp 56 Asp 57 Phe 58 Lys 59 Tyr 60 Asn 61 Glu 62 Arg 63 Phe 64 Lys 65 Gly

CDR 3  
HuCC49  
21/28'CL  
95 Ser 96 Leu 97 Asn 98 Met 99 Ala 100 Ser 101 Asn 102 Tyr

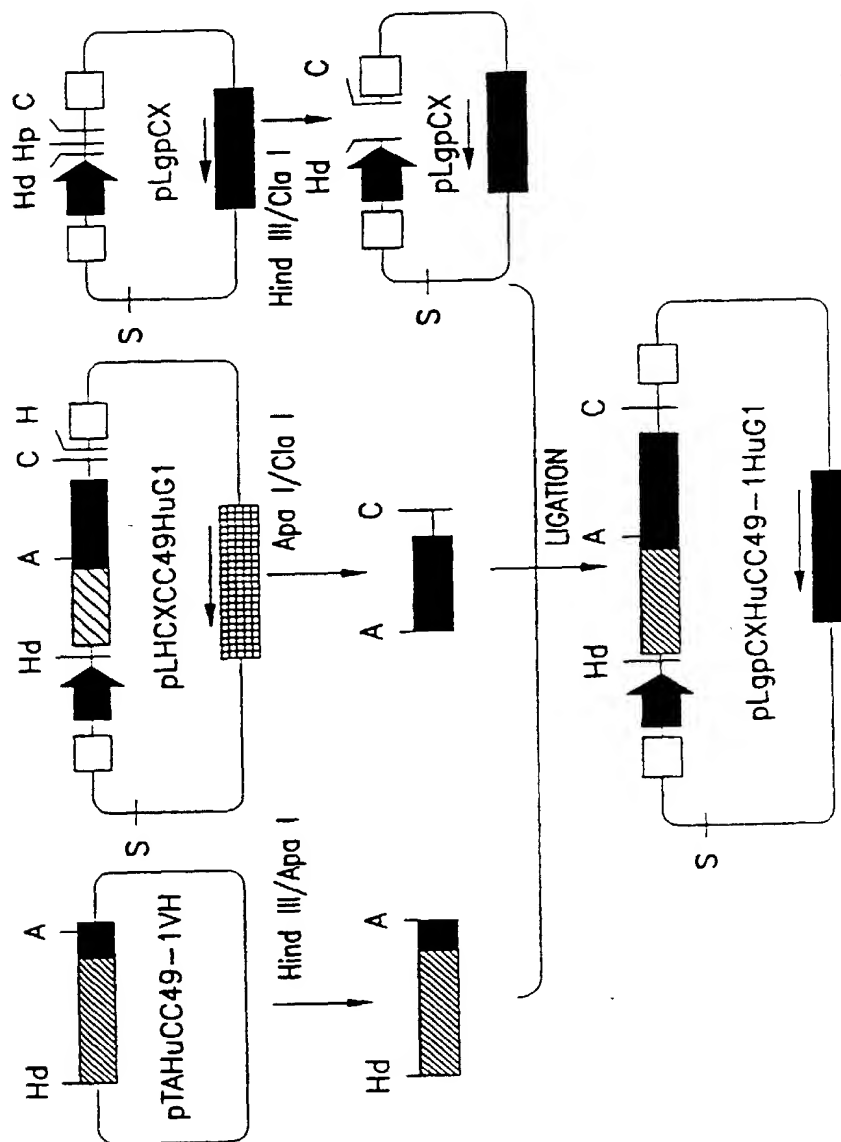


FIG. 3

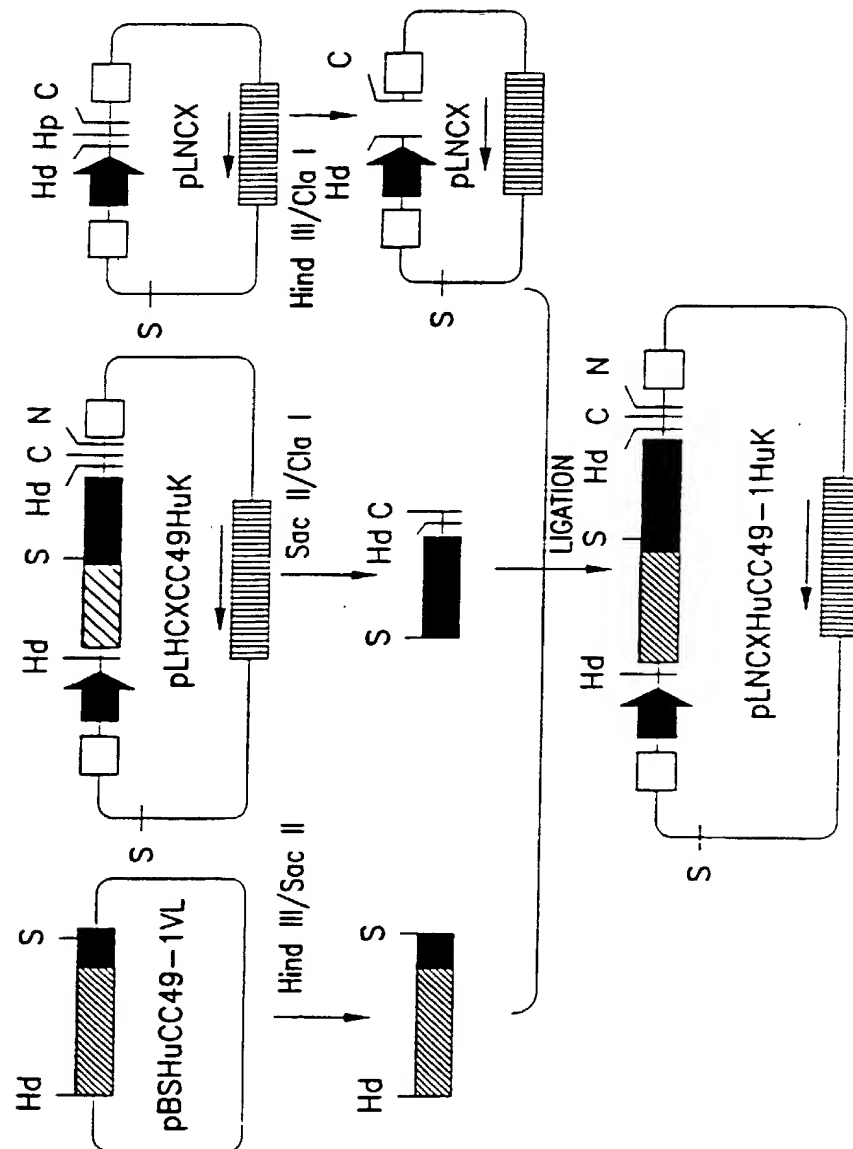
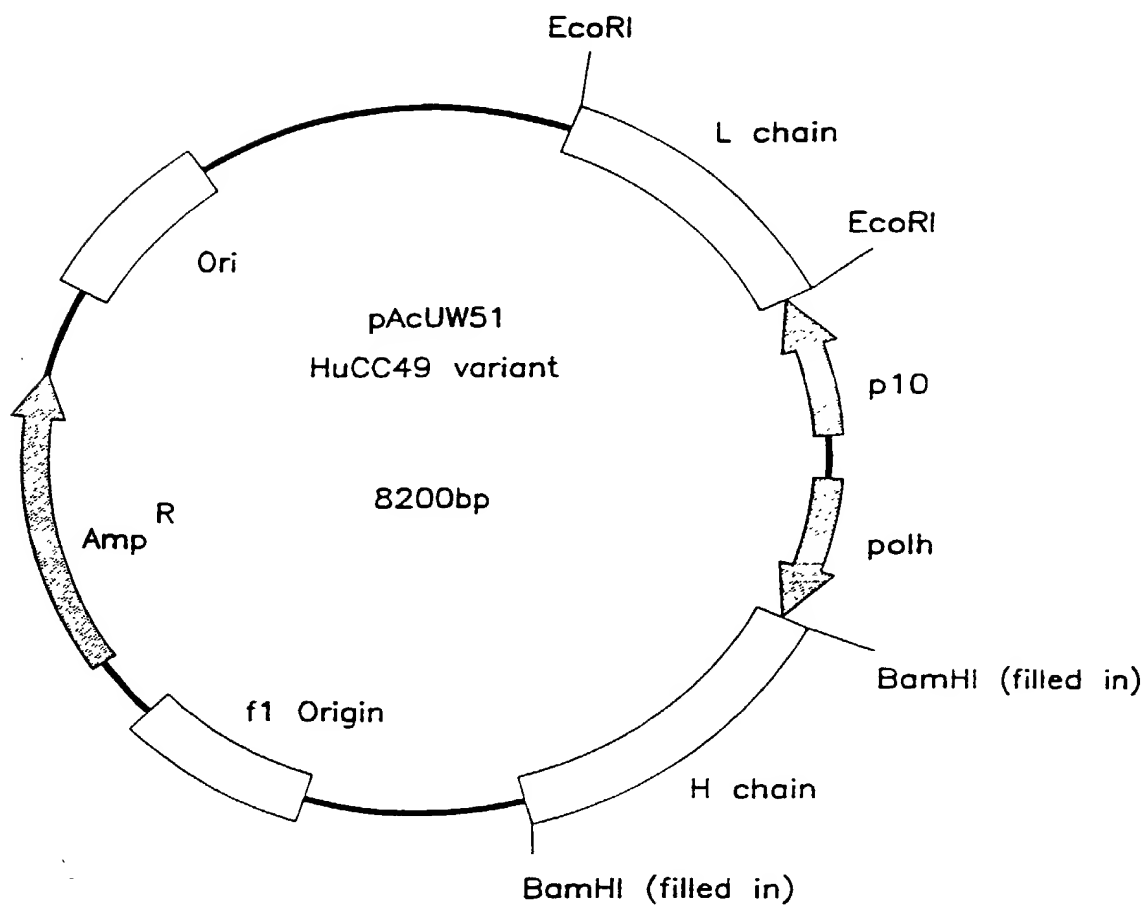


FIG. 4

FIG. 5





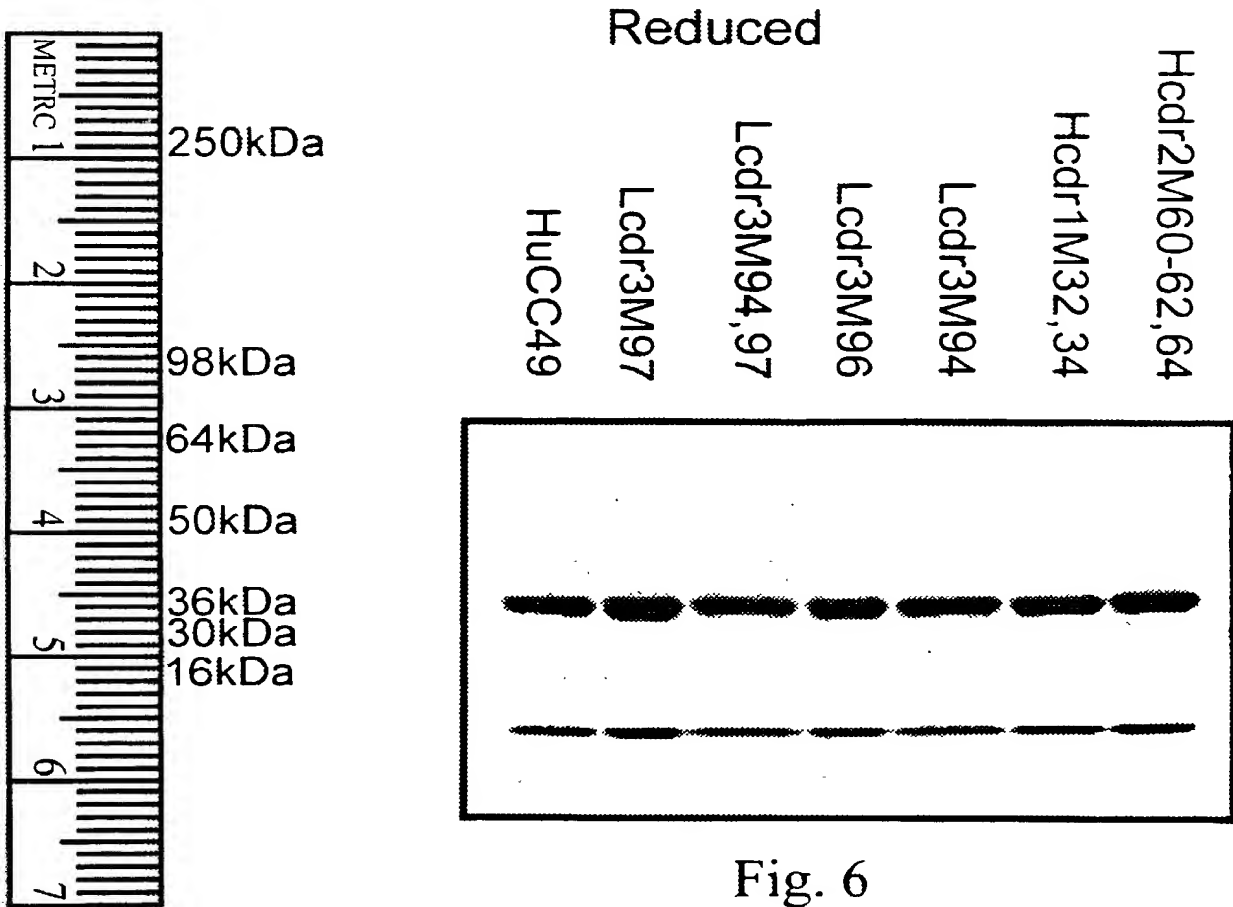


FIG. 7

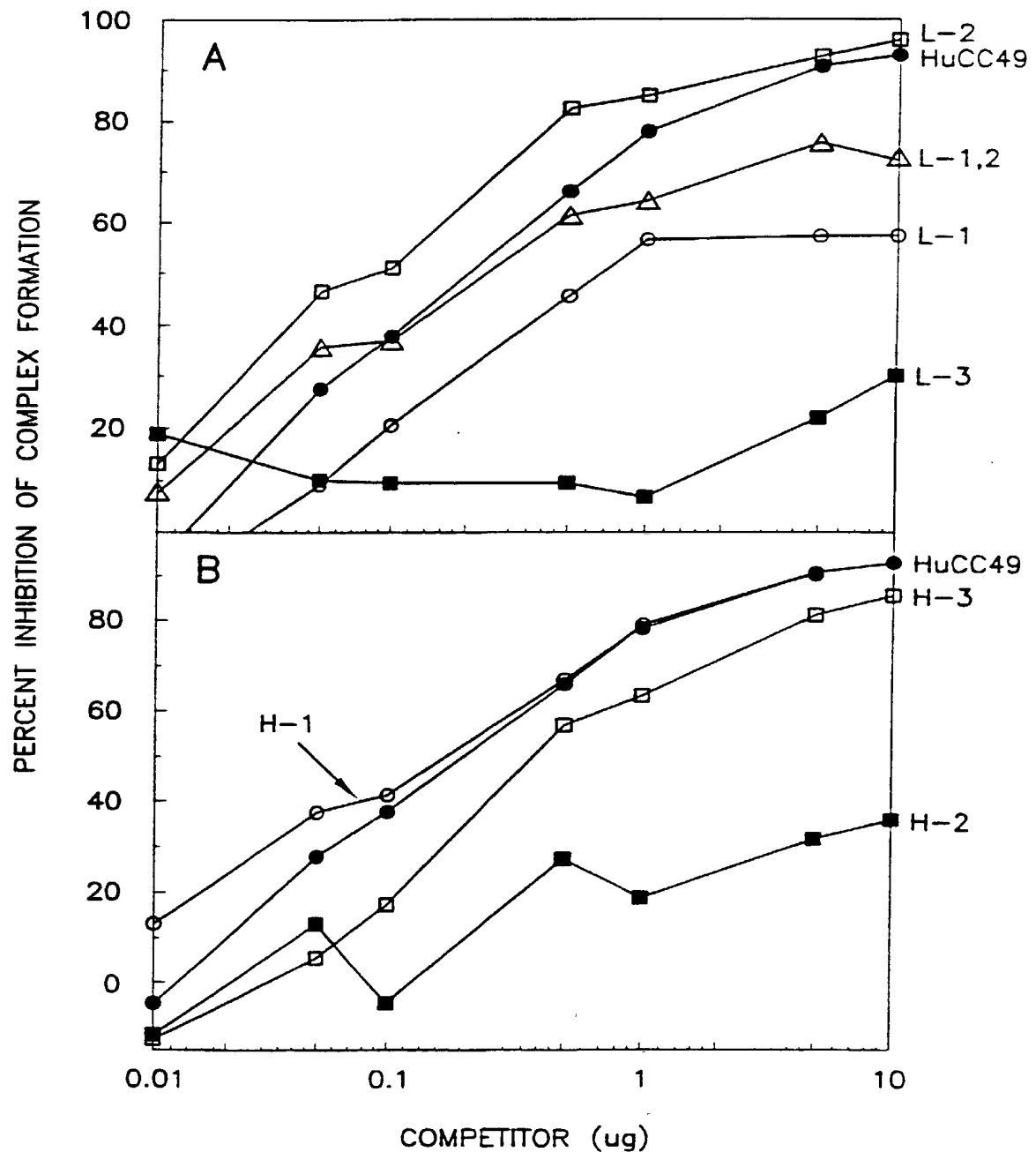
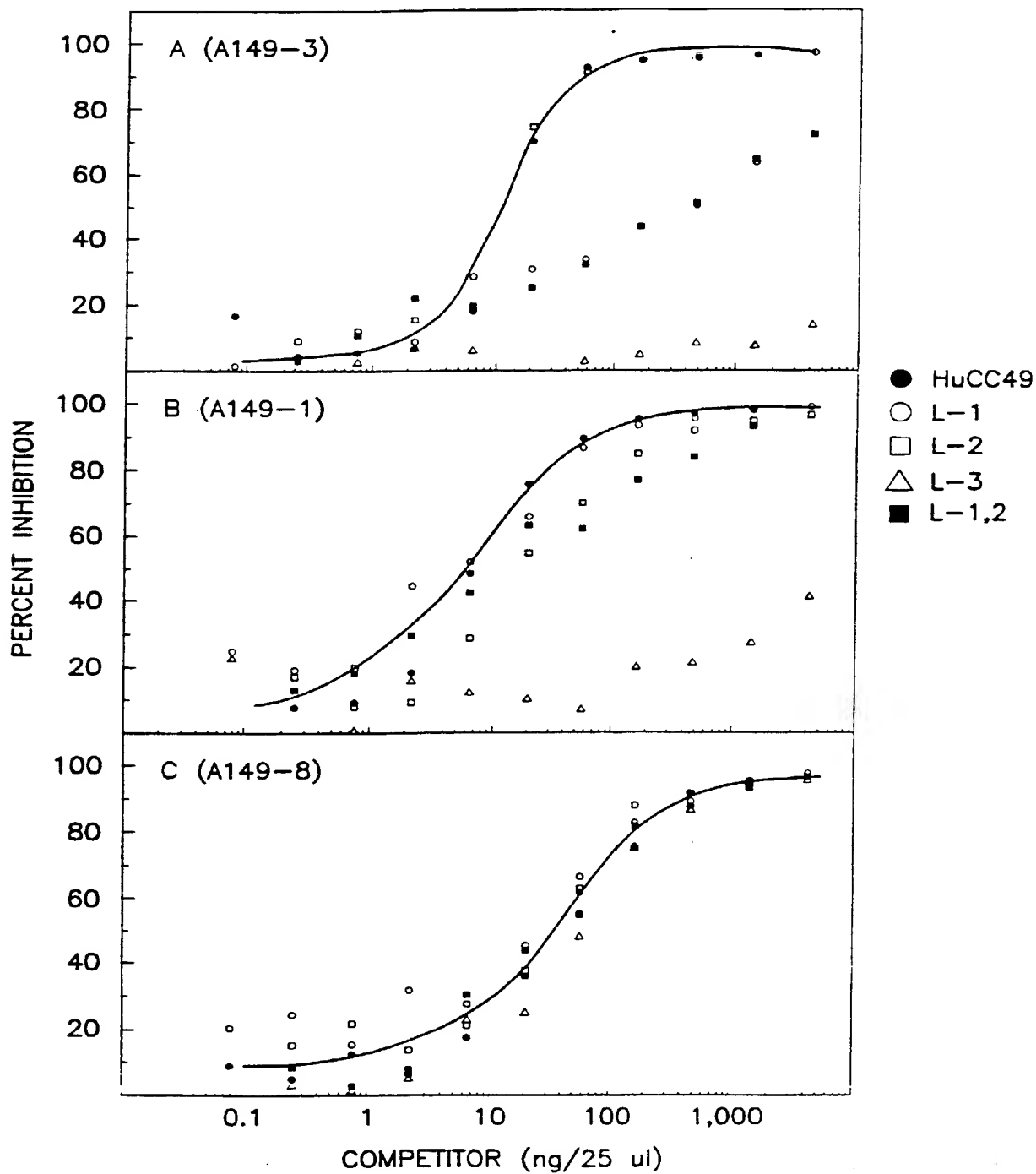


FIG. 8



9/23

FIG. 9

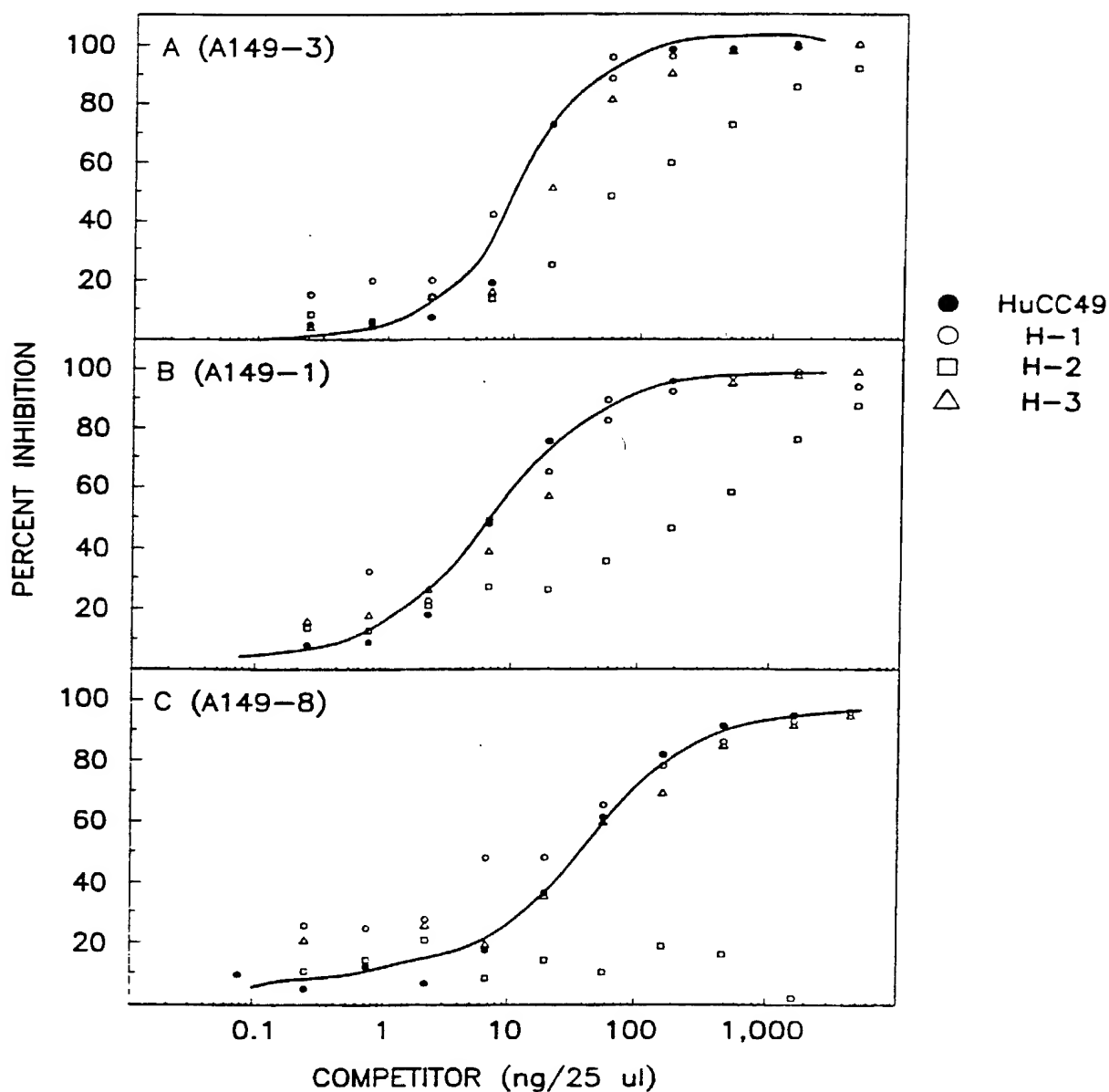


FIG. 10

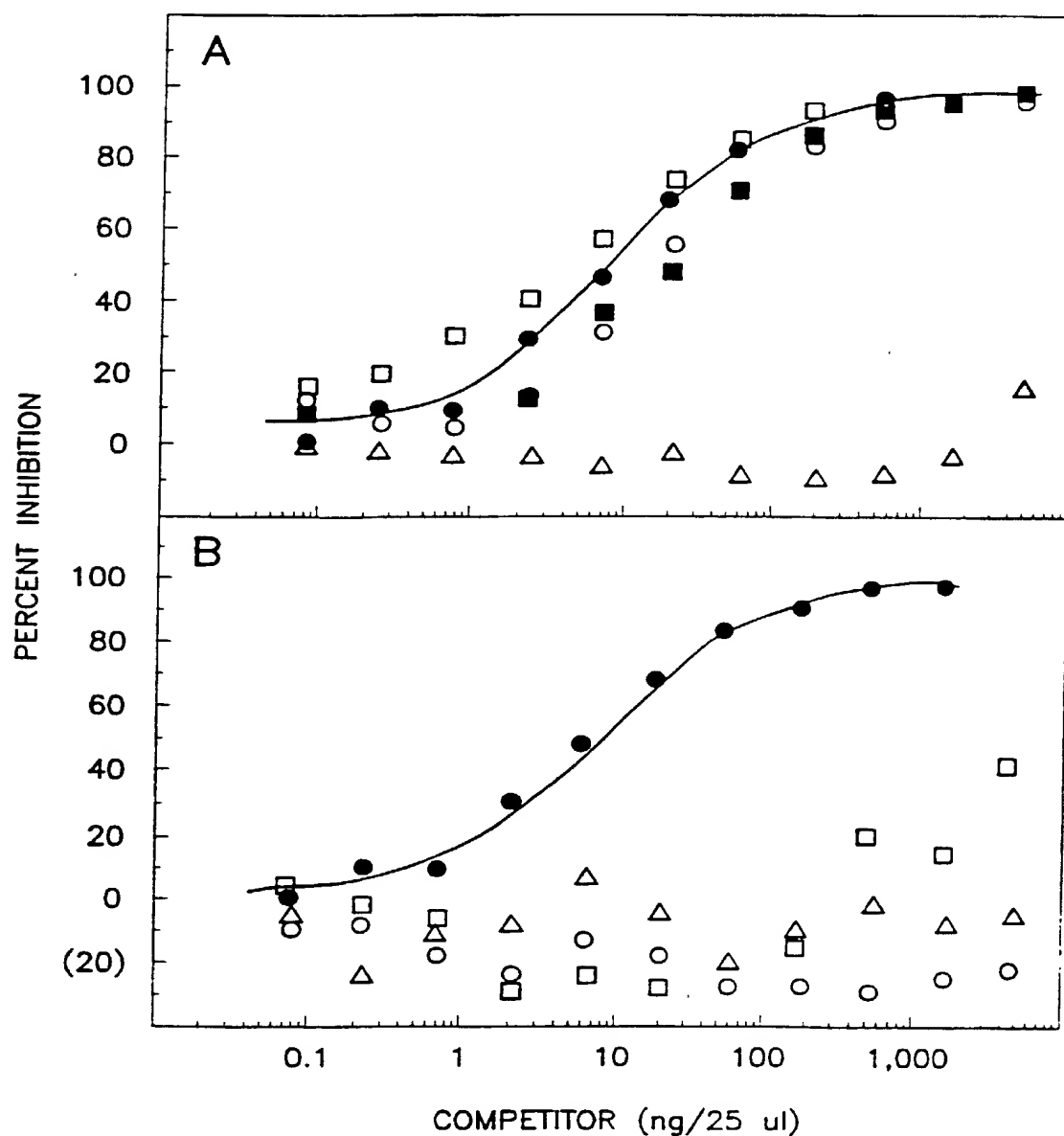


FIG. 11

A.

LEN HuCC49	DIVMTQSPD DIVMSQSPD	SLAVSLGERATINC SLAVSLGERVTLC	KSSQSLLYSGNQKNYLA	WYQKPGQPPKLLIY WYQKPGQSPKLLIY	CDR1	CDR2	CDR3	FGQGTKLEIK FGAGTKLELK
LEN HuCC49	GVPDRFSGSGGTDFTLTIS	SLQAEDVAVYYC	WASARES	GVPDRFSGSGGTDFTLTIS	SVQAEDVAVYYC	QYYYSYPLT	FGQGTKLEIK FGAGTKLELK	

B.

21/28'CL HuCC49	QVQLVQSGAEVKKPGASVKV	SCKASGYTFT	DHAIH	WVRQAPGQRLEWMG	CDR1	CDR2	CDR3
21/28'CL HuCC49	RVTIITRDTSASTAYMELSS	LSRSEDIAVYYCAR	YFSPGNDDFKYNERFKG	KATLTADTSASTAYVELSS	LSRSEDIAVYFCTR	WGQGTILVTVSS	SLNMAY WGQGTILVTVSS

WO 00/26394

FIG. 12A

A.

```
1      gcaagcttccaccATGGATAGCCAGGCCAGGTGCTCATGCTCCTGCTGCTGTGGGTGAG
60      +-----+-----+-----+-----+-----+-----+
      cgttcgaaggtggTACCTATCGGTCCGGTCCACGAGTACGAGGACGACGACCCCACTC
120      CGGCACATGCGGCGGACATCGTGATGAGCCAGTCTCCAGACTCCCTGGCCCGTGTCCCAAGG
      +-----+-----+-----+-----+-----+-----+
      GCCGTGTACGCCCGCTGTAGCACTACTCGGTCAAGGCTCTGAGGACCGGCACAGGGACCC
180      CGAGAGGGTGACTCTGAATTGCAAGTCCAGCCAGTCCCTGCTCTATAGCGGAAATCAGAA
      +-----+-----+-----+-----+-----+-----+
      GCTCTCCCACTGAGACTTAACGTTACGTTCCGGTCCAGGACGAGATATCGCCCTTTAGTCTT
240      GAACTATCTCGCCCTGGTATCAGCAGAAACCAGGGCAGAGCCCTAAACTGCTGATTTACTG
      +-----+-----+-----+-----+-----+-----+
      CTGTATAGAGCGGACCATAGTCGTCTTGGTCCCGTCTCGGGAATTGACGACTAAATGAC
300      GGCATCCGCTAGGGAAATCCGGCGTGCCCTGATCGCTTCAGCGGCAGCGGATCTGGGACAGA
      +-----+-----+-----+-----+-----+-----+
      CCGTAGGCGATCCCTTAGGCCCGCACGGACTAGCGAAGTCGCCGTAGACCCCTGTCT
360      CTTCACCTCTGACAAATCAGCAGCGTGCAGGCGCAGAGACGTGGCAGTCTATTATTGTCAACA
      +-----+-----+-----+-----+-----+-----+
      GAAGTGAGACTGTTAGTCGTCCGACGTCCGTCCTTCTGCACCCGTCAGATAATAACAGTCGT
420      GTATTATAGCTATCCCCCTCACATTCGGCGCTGGCACCAAGCTGGAAGTGAACggcg
      +-----+-----+-----+-----+-----+-----+
      CATAATATCGATAGGGGAGTGTAAAGCCCGGACCCGTGGTTCGACCTTGACTTTgcccggcg
      ggct      424
      +-----+
      ccga
```

**FIG. 12B**

m.

1      c t a a g c t t c c a c c a t g g a g t g g t c c t g g g t c t t c c t c t t c c t g c t g c t g t g g g t g a g  
 60      g a t t c g a a g g t g g t a c c t c a c c a g g a c c c a g a a g g a g a g g a c g a c g a c a c c a c t c  
 120      a g t g c a c t c c c a g g t c c a g c t g g t g c a g t c c g g c t g a g t c c c t g g c c g t g t c c c a g g g  
 180      t c a c g t g a g g g t c c a g g t c g a c c a c g t c a g g c c g c g a c t c a g g a c c g g c a c a g g g a c c c  
 240      c g t g a a g a t t t c c t g c a a g g c a a g c g g c t a c a c c t t c a c t c t c t a t a g c g g a a a t c a g a a  
 300      g c a c t t c t a a a g g a c g t t c c g t t c g c c g a t g t g g a a g t g a g a g a t a t c g c c t t t a g t c t t  
 360      g a a a c a g a a t c c t g g a c a g c c c t g g a g t g g a t t g g a t a t t t c t c t c c c g g a a a c g a t g a  
 420      c t t t g t c t t a g g a c c t g t c g c g g a c c t c a c c t a a c c t a t a a g a g a g g c c t t t g c t a c t  
 480      t t t t a a g t a c a a t g a g a g g t t c a a g g c a a g g c c a c a c t g a c t g c a g a c a c a t c t g c c a g  
 540      a a a a t t c a t g t t a c t c t c c a a g t t c c c g t t c c g g t g t g a c t g a c g t c t g t g t a g a c g g t c  
 600      c a c t g c c t a c g t g g a g c t c t c c a g c c t g a g a t c c g a g g a t a c t g c a g t g t a c t t c t g c a c  
 660      g t g a c g g a t g c a c c t c g a g a g g t c g g a c t c t a g g c t c c t a t g a c g t c a c a t g a a g a c g t g  
 720      a a g a t c c c t g a a t a t g c c t a c t g g g c a c a g g g a a c c c t g g t c a c c g t c t c c a g c g c c a a  
 780      t t c t a g g g a c t t a t a c c g g a t g a c c c c t g t c c c t t g g g a c c a g t g g c a g a g g t c g c g g t t  
 840      a a c t a c g g g c c c a t  
 900      t t g a t g c c c g g g t a



FIG. 13

COMPETITION RADIOIMMUNASSAY:  
MANIPULATED CDR VARIANTS OF HuCC49

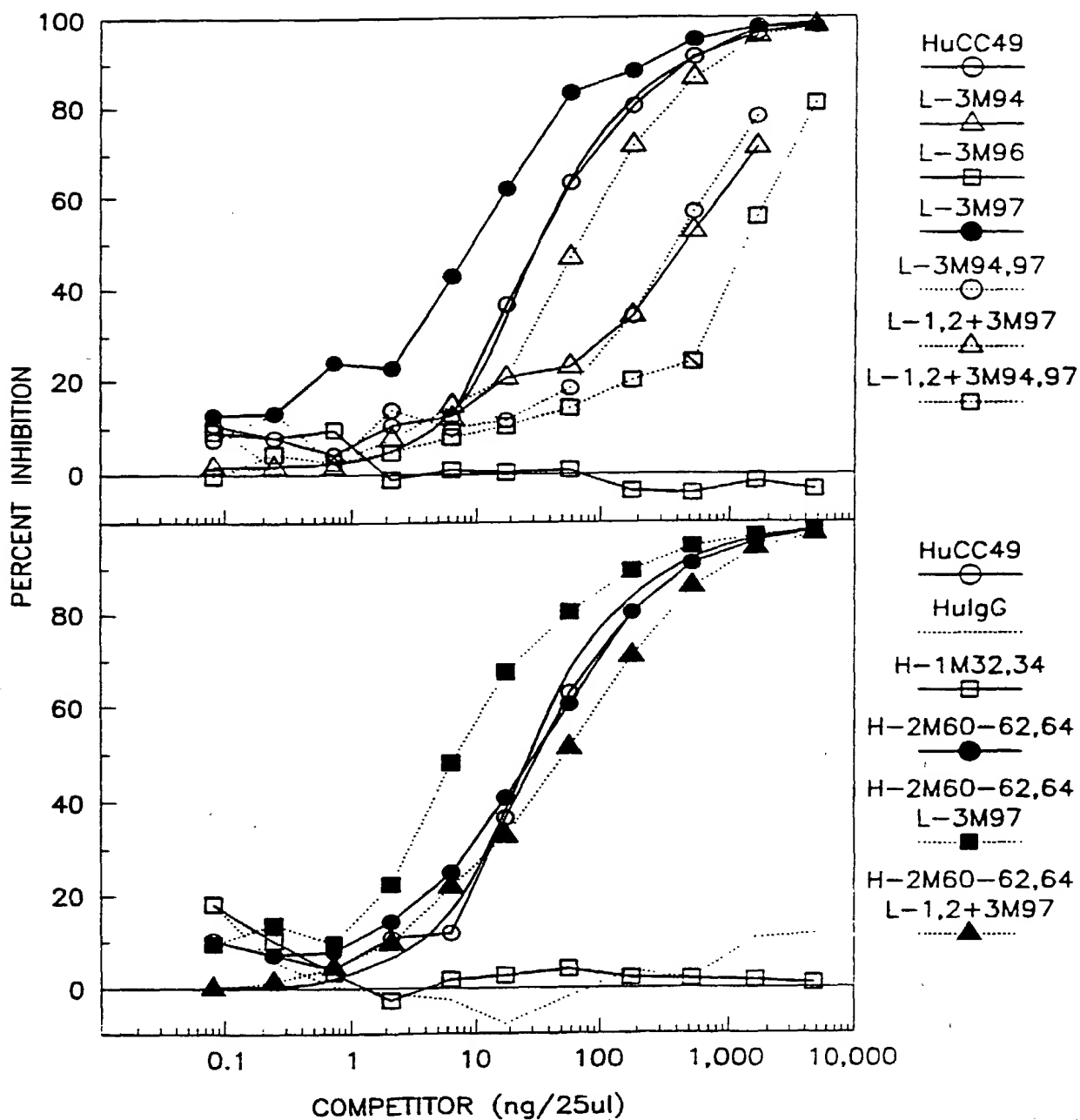


FIG. 14

HPLC ANALYSIS OF PATIENT REACTIVITY TO CDR SUBSTITUTION  
VARIANTS OF HuCC49

COMPETITOR			PATIENTS			
	CDR SUBSTITUTIONS	ANTIGEN BINDING	DG	CP	EA	DS
NONE	---		46.2 <sup>b</sup>	32.2	56.8	33.5
HuCC49	---	+++	0	1.5	0.5	0
HuIgG	---	-	59.0	N.D.	63.6	46.4
LIGHT	L3M94	+/-	30.2	20.3	16.4	28.9
	L3M96	-	39.2	31.1	42.9	35.2
	L3M97	+++	0.6	1.3	0.7	2.4
	L3M94,97	+/-	26.5	18.2	18.6	25.6
	L1,2+3M97	++	21.3	17.6	23.8	17.1
	L1,2+3M94,97	+	53.2	38.1	44.2	37.3
HEAVY	1M32,34	-	1.4	5.5	3.8	0.7
	2M60-62,64	++	24.4	17.9	21.8	16.5
BOTH	L3M97	++++	13.0	16.1	3.9	20.1
	H2M60-62,64					
	L1,2+3M97	++	33.0	30.7	24.9	32.1
	H2M60-62,64					

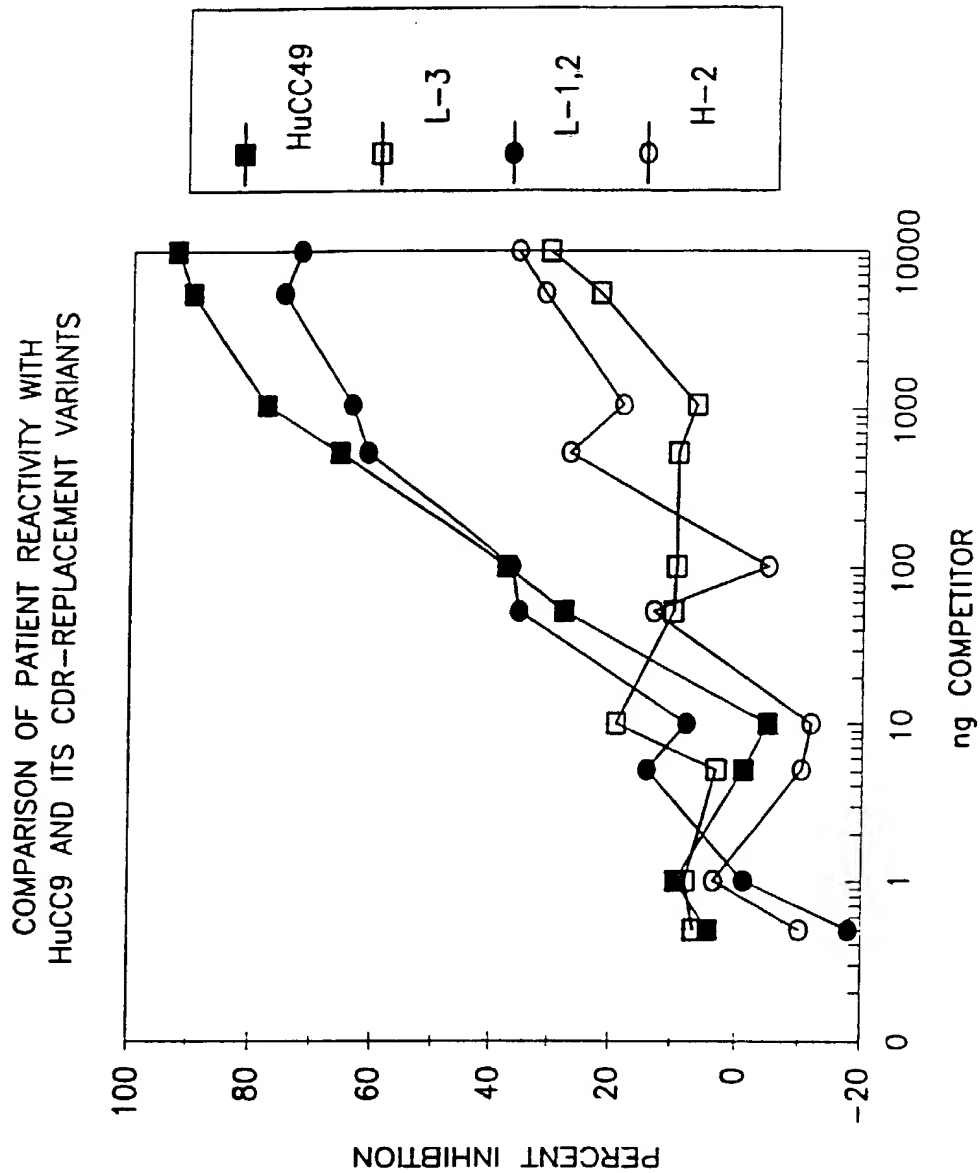


FIG. 15

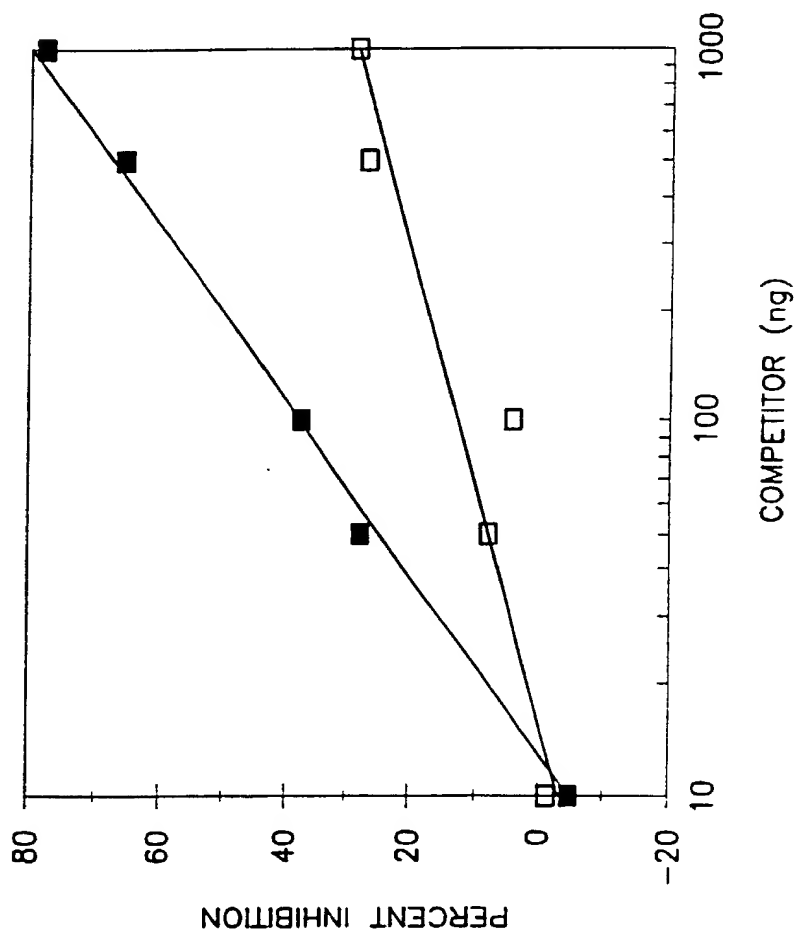


FIG. 16

PHARMACOKINETICS OF PLASMA RETENTION OF  
RADIOIODINATED HuCC49 AND VARIANT

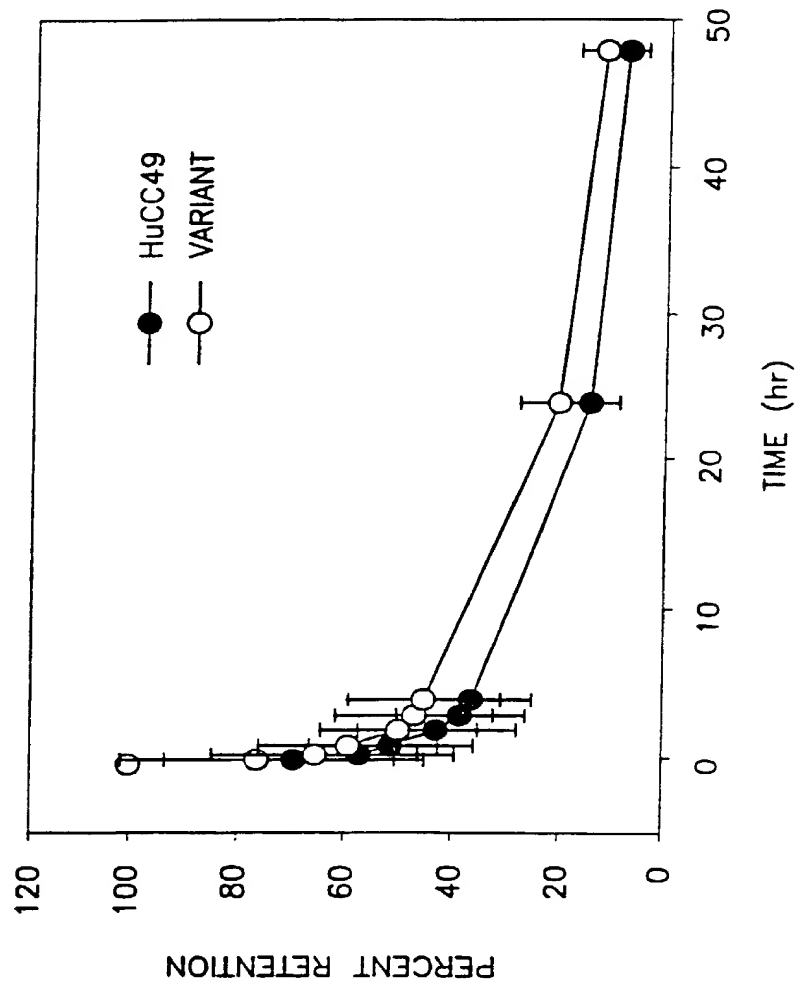


FIG. 17

FIG. 18

BIODISTRIBUTION OF I.V. ADMINISTERED RADIOLABELED HuCC49 AND VARIANT IN ATHYMIC MICE BEARING LS-174T HUMAN COLON CARCINOMA XENOGRAFTS: PERCENT OF INJECTED DOSE/GRAM

ANTIBODY	ORGAN	TIMEPOINTS (hr)				
		24	48	72	120	168
VARIANT	TUMOR	15.83	23.75	21.01	17.74	9.21
	BLOOD	6.35	4.93	4.88	2.19	0.63
	LIVER	3.39	2.14	1.46	0.91	0.32
	SPLEEN	5.90	6.04	2.55	2.43	3.96
	KIDNEY	2.52	1.27	1.00	0.77	0.36
	LUNG	3.22	2.57	2.50	1.12	0.36
HuCC49	TUMOR	11.86	17.59	15.31	13.75	5.24
	BLOOD	4.17	2.94	2.85	1.29	0.18
	LIVER	4.77	3.05	1.41	0.70	0.12
	SPLEEN	6.41	7.47	2.28	2.00	0.46
	KIDNEY	1.86	0.92	0.70	0.57	0.14
	LUNG	2.17	1.58	1.46	0.68	0.12

FIG. 19

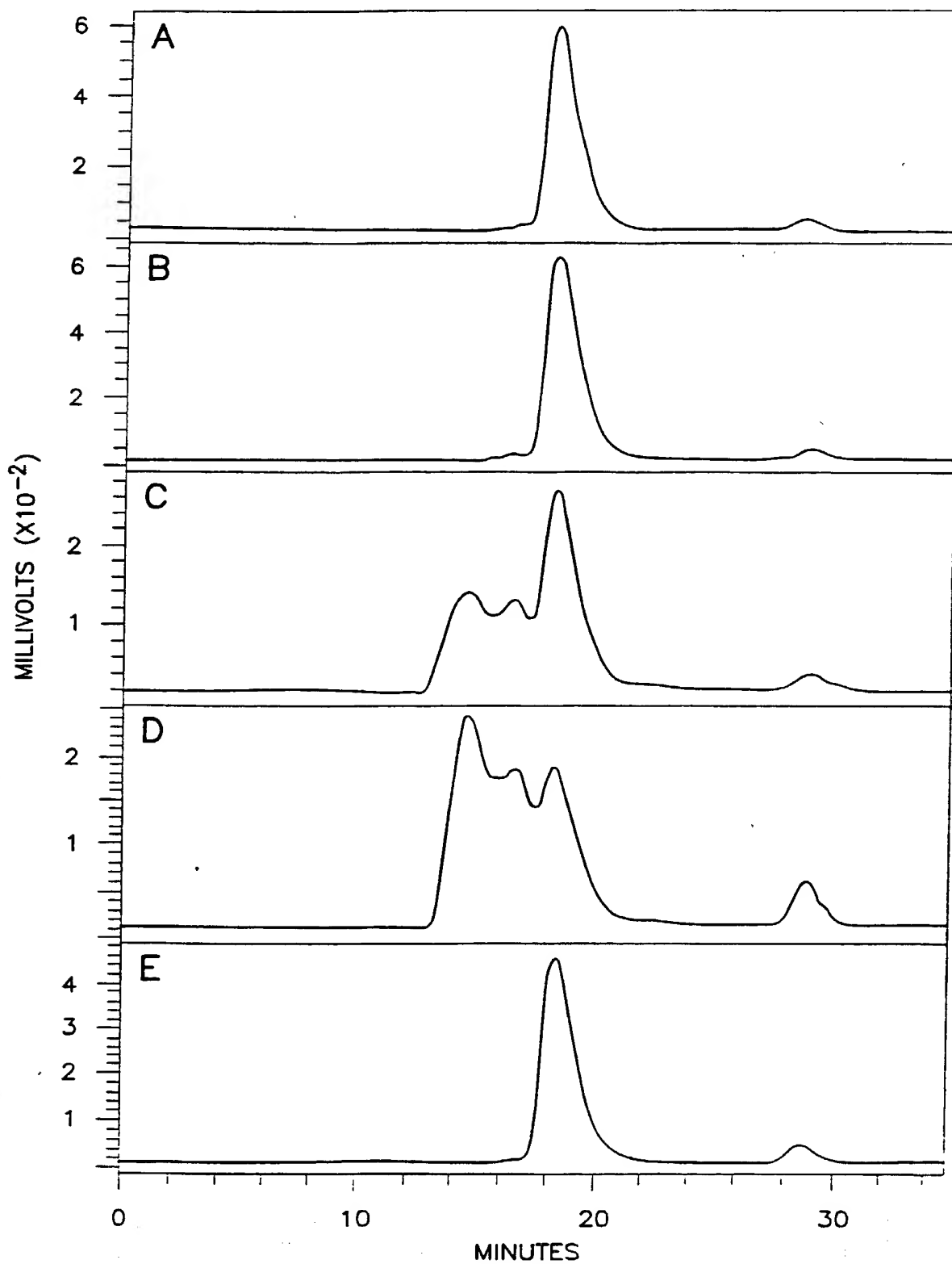
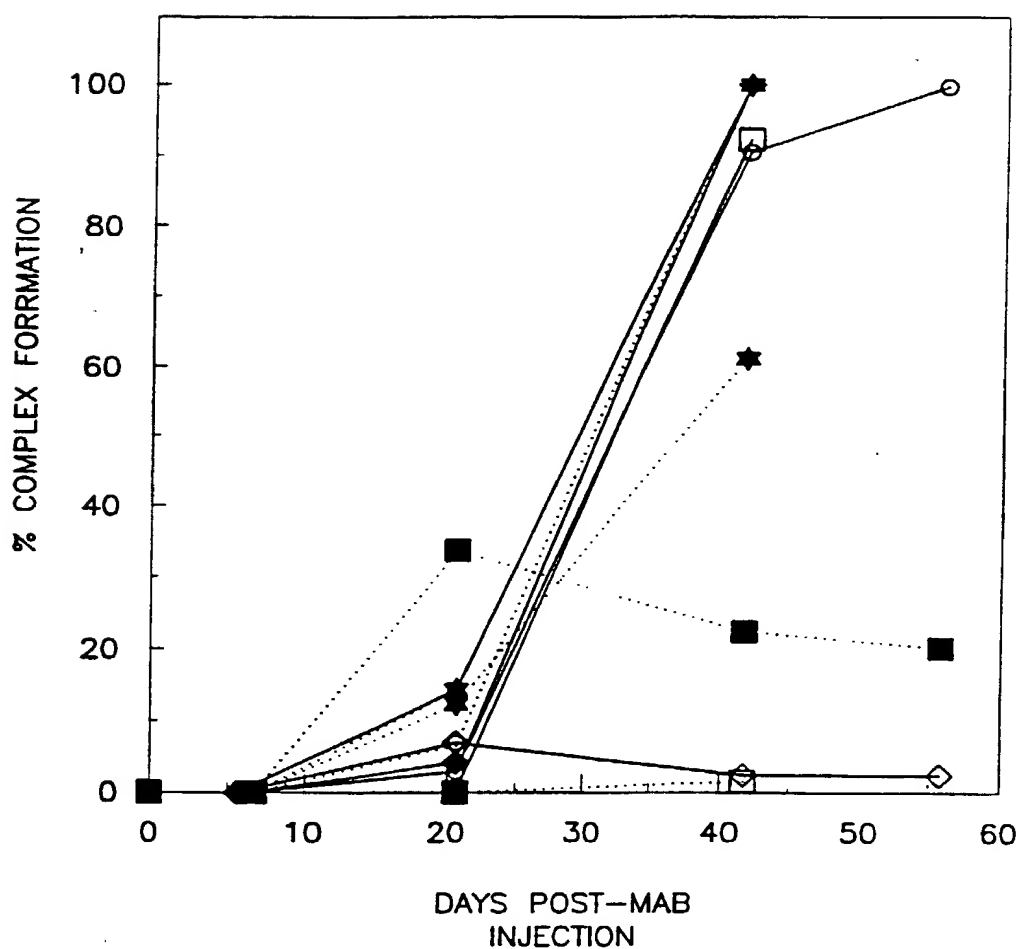


FIG. 20





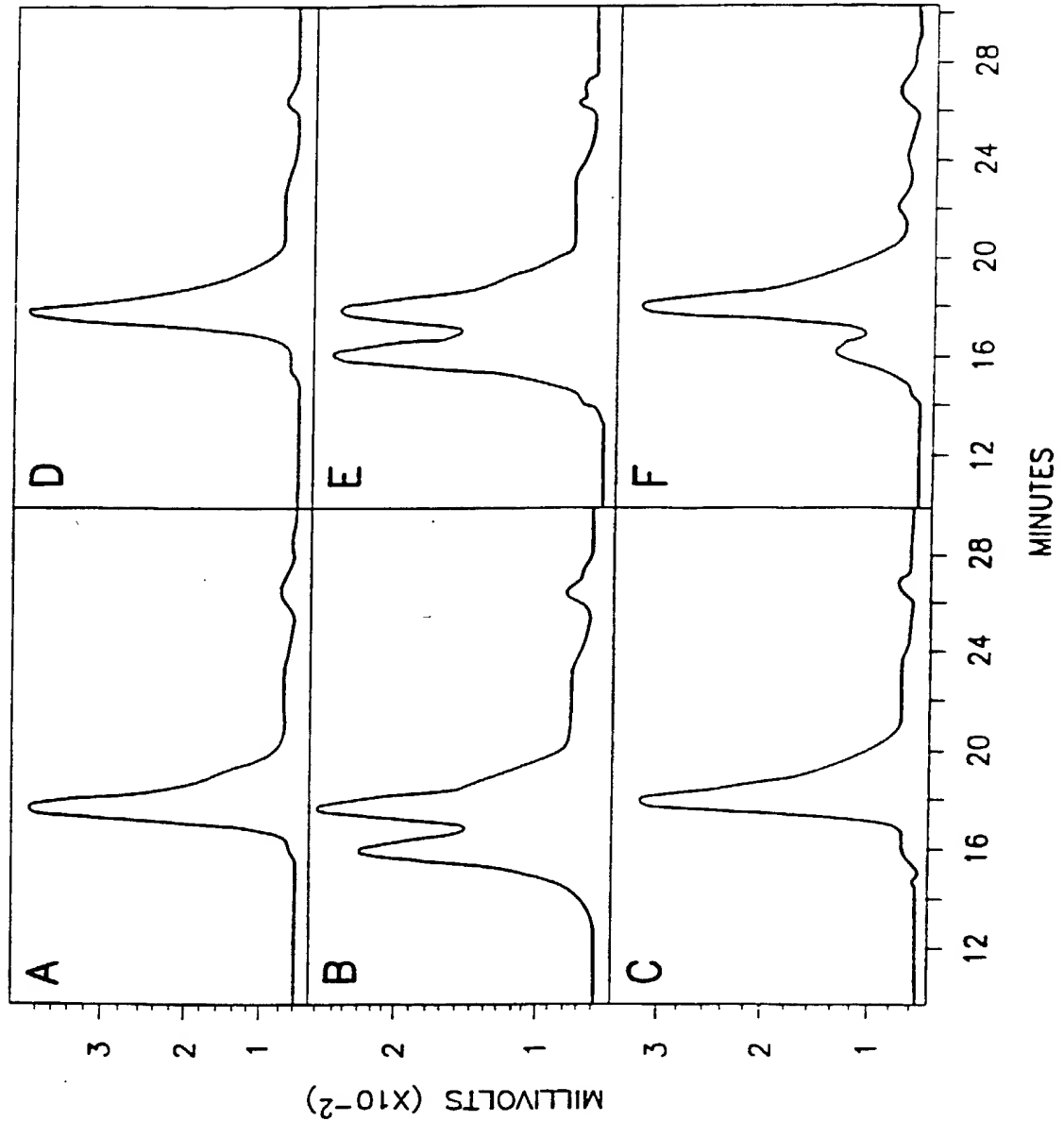


FIG. 21

WO 00/26394

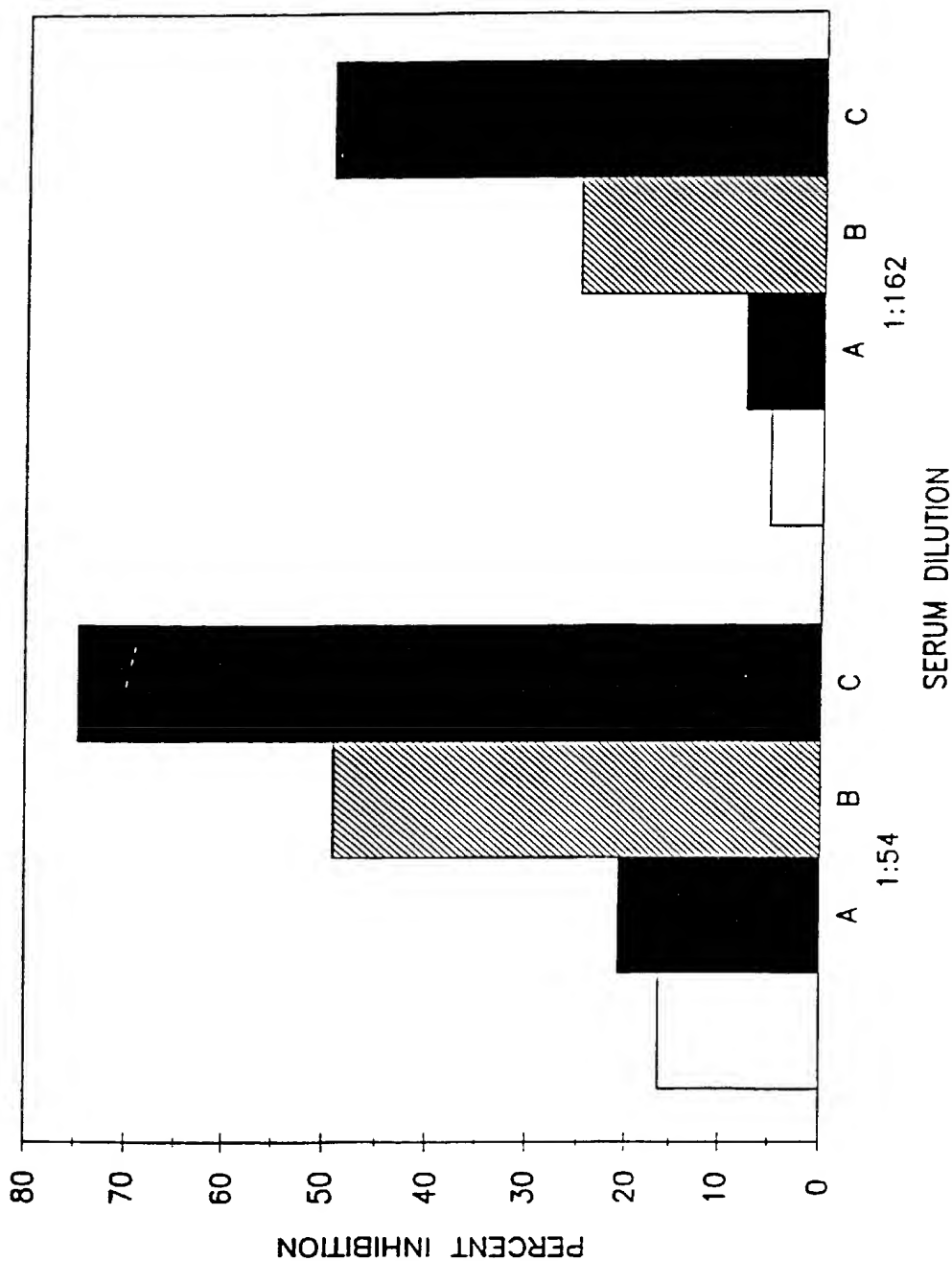


FIG. 22

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Kashmiri et al.

Art Unit: Not yet assigned

Application No. 09/830,748

Filed: April 30, 2001

For: VARIANTS OF HUMANIZED ANTI-  
CARCINOMA MONOCLONAL  
ANTIBODY CC49

Examiner: Not yet assigned

Date:

CERTIFICATE OF FACSIMILE TRANSMISSION

I hereby certify that this correspondence and any documents referred to as being attached or enclosed herewith are being transmitted to the United States Patent and Trademark Office, to the attention of Receptionist, Box PCT, U.S. Patent and Trademark Office, at the facsimile telephone number (703) 305-3230, on 8 Jan 2002

William D. Noonan  
William D. Noonan, M.D.  
Attorney for Applicant

**REVOCATION OF PRIOR POWER OF ATTORNEY  
AND NEW POWER OF ATTORNEY BY ASSIGNEE AND  
CERTIFICATE BY ASSIGNEE UNDER 37 CFR § 3.73**

COMMISSIONER FOR PATENTS  
WASHINGTON D.C. 20231

Dear Sir:

THE GOVERNMENT OF THE UNITED STATES OF AMERICA, AS REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES, the Assignee of the entire right, title and interest in the above-identified patent application, by Assignment attached hereto, and recorded at Reel 010810, Frame 0055, recorded on July 25, 2000, hereby revokes all prior powers of attorney and instead appoints as its representatives to conduct all business in the Patent and Trademark Office connected therewith the attorneys and agents identified below:

Name	Reg. No.	Name	Reg. No.
James C. Haight	<u>25,588</u>	Jack Spiegel	<u>34,477</u>
Robert Benson	<u>33,612</u>	David R. Sadowski	<u>32,808</u>
Susan S. Rucker	<u>35,762</u>	Steve Ferguson	<u>38,448</u>
John Peter Kim	<u>38,514</u>	Richard U. Rodriguez	<u>45,980</u>
Stephen L. Finley	<u>36,357</u>	Marlene Shinn	<u>46,005</u>
Norbert Pontzer	<u>40,777</u>		

all of the National Institutes of Health, Office of Technology Transfer, 6011 Executive, Boulevard, Suite #325, Rockville, MD 20852; and grants an Associate Power of Attorney to:

Name	Reg. No.	Name	Reg. No.
Richard J. Polley	<u>28,107</u>	Lisa M. Caldwell	<u>41,653</u>
William D. Noonan	<u>30,878</u>	Michael D. Jones	<u>41,879</u>
Donald L. Stephens Jr.	<u>34,022</u>	Tanya M. Harding	<u>42,630</u>
Stacey C. Slater	<u>36,011</u>	Gregory L. Maurer	<u>43,781</u>

Susan Alpert Siegel      43,121  
Wayne W. Rupert      34,420-

Robert F. Scotti      39,830  
Sheree L. Rybak      47,913

all of the law firm of Klarquist Sparkman, LLP, 121 SW Salmon Street, Suite 1600, Portland, Oregon 97204.

The Assignee certifies that the above-identified Assignment has been reviewed and to the best of Assignee's knowledge and belief, title is in the Assignee, as demonstrated by the copy of the Assignment submitted herewith.

Please address all correspondence to William D. Noonan, M.D. at:

KLARQUIST SPARKMAN, LLP  
One World Trade Center, Suite 1600  
121 SW Salmon Street  
Portland, OR 97204-2988  
Telephone: 503-226-7391  
Fax: 503-228-9446

The National Institutes of Health Office of Technology Transfer has been duly delegated responsibility for patent matters under the authority of the memorandum dated May 21, 1991, from Louis W. Sullivan, the Secretary of the Department of Health and Human Services, delegating the authority to the Heads of the Public Health Service Operating Divisions, as well as a memorandum dated October 9, 2001, from Mark L. Rohrbaugh, Ph.D., J.D., Acting Director, Office of Technology Transfer, National Institutes of Health, delegating authority to sign this Power of Attorney to the undersigned individual. The undersigned is therefore authorized to exercise such authority in this matter.

Executed on 12-11-01, at Rockville, Maryland.

UNITED STATES DEPARTMENT OF  
HEALTH AND HUMAN SERVICES

Richard U. Rodriguez  
Name: Richard U. Rodriguez  
Reg. No.: 45,980  
National Institutes of Health  
Office of Technology Transfer  
6011 Executive Boulevard, Suite #325  
Rockville, MD 20852

## ASSIGNMENT

WHEREAS, we, Syed V.S. Kashmiri, residing at 11541 Brandy Hall Lane, Gaithersburg, Maryland 20878, Eduardo A. Padlan, residing at 4006 Simms Drive, Kensington Maryland 20895, and Jeffrey Schlom, residing at 10301 Sorrel Avenue, Potomac, Maryland 20854, employees of the Department of Health and Human Services, have invented VARIANTS OF HUMANIZED ANTI-CARCINOMA MONOCLONAL ANTIBODY CC49 for which filed an international application, PCT US99 25552, on October 29, 1999, designating the United States as one of the Designation of States, and

WHEREAS, we are applicants named in the above-mentioned application for Letters Patent; and

WHEREAS, the conditions under which said invention was made are such as to entitle the Government under Paragraph 1(a) of Executive Order 10096 to the entire right, title, and interest herein, both domestic and foreign, and

WHEREAS, the Government of the United States is desirous of acquiring all domestic and foreign right, title, and interest in the above-mentioned invention described in the application for Letters Patent; and

NOW, THEREFORE, for good and valuable consideration the receipt of which is hereby acknowledged, we hereby assign and transfer to the United States of America, represented by the Secretary, Department of Health and Human Services, the full and exclusive rights in and to said invention in the U.S. and within each and every foreign country in which the Government elects to file and the entire right, title, and interest in and to such applications, and any continuations, continuations-in-part, divisions, reissues or extensions thereof, and including priority rights as may be filed in the U.S. and foreign countries, and such Letters Patent as may be granted to be held by the Government to the end of the term for which the same would have been held by the inventors had this assignment not been made

We further agree to make, execute, and deliver to the Secretary, Department of Health and Human Services, upon request, any and all papers, documents, affidavits, or other instruments that may be necessary in the prosecution of any application or applications for improvements or reissues of Letters Patent, and to assist the Government in every way as may be requested in protecting said invention, provided that any expense of extending such assistance shall be paid by the Government

IN TESTIMONY WHEREOF, I have hereunto set my hand this \_\_\_\_\_ day of

Jan 19, 192000

S. V. S. Kashmiri  
Syed V.S. Kashmiri

STATE OF Maryland )  
COUNTY OF Montgomery ) ss.

MY COMMISSION EXPIRES  
MARCH 1, 2003  
MARGARET L. TOMCZAK  
NOTARY PUBLIC

On this 19th day of January, 192000, before me personally appeared Syed V S Kashmiri to me known and known to me to be the person described in and who executed the foregoing instrument, and he duly acknowledged to me that he executed the same for the uses and purposes therein set forth.

[SEAL]

Margaret L. Tomczak  
Notary Public

IN TESTIMONY WHEREOF, I have hereunto set my hand this 18 day of

January, 192000

Eduardo A. Padlan  
Eduardo A. Padlan

STATE OF Maryland )  
COUNTY OF Montgomery ) ss.

On this 18th day of January, 192000, before me personally appeared Eduardo A. Padlan to me known and known to me to be the person described in and who executed the foregoing instrument, and he duly acknowledged to me that he executed the same for the uses and purposes therein set forth.

[SEAL]

Margaret L. Tomczak  
Notary Public

MY COMMISSION EXPIRES  
MARCH 1, 2003  
MARGARET L. TOMCZAK  
NOTARY PUBLIC

IN TESTIMONY WHEREOF, I have hereunto set my hand this 18 day of

Jan, 192000

Jeffrey Schlom  
Jeffrey Schlom

STATE OF Michigan )  
COUNTY OF Macomb ) ss

On this 18th day of January, 2000 before me personally appeared Jeffrey Schlom to me known and known to me to be the person described in and who executed the foregoing instrument, and he duly acknowledged to me that he executed the same for the uses and purposes therein set forth.

(SEAL)

Margaret L. Tomczak  
Notary Public

MY COMMISSION EXPIRES  
MARCH 1, 2003  
MARGARET L. TOMCZAK  
NOTARY PUBLIC

Attorney Docket No. 11613.32USWO

MERCHANT &amp; GOULD P.C.

## United States Patent Application

## COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that

I verily believe I am the original, first and sole inventor (if only one name is listed below) or a joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: VARIANTS OF HUMANIZED ANTI-CARCINOMA MONOCLONAL ANTIBODY CC49

The specification of which

a. ☐ is attached hereto

b. ☒ was filed on as application serial no. and was amended on (if applicable) (in the case of a PCT-filed application) described and claimed in international no. PCT/US99/25552 filed 29 October 1999 and as amended on (if any), which I have reviewed and for which I solicit a United States patent.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on the basis of which priority is claimed:

a. ☐ no such applications have been filed.

b. ☒ such applications have been filed as follows:

FOREIGN APPLICATION(S), IF ANY, CLAIMING PRIORITY UNDER 35 USC § 119			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)
ALL FOREIGN APPLICATION(S), IF ANY, FILED BEFORE THE PRIORITY APPLICATION(S)			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)

I hereby claim the benefit under Title 35, United States Code, § 120/365 of any United States and PCT international application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. APPLICATION NUMBER	DATE OF FILING (day, month, year)	STATUS (patented, pending, abandoned)

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below:

U.S. PROVISIONAL APPLICATION NUMBER	DATE OF FILING (Day, Month, Year)
60/106534	31 October 1998
60/106757	02 November 1998



I acknowledge the duty to disclose information that is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56 (reprinted below):

**§ 1.56 Duty to disclose information material to patentability.**

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is canceled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§ 1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

- (1) prior art cited in search reports of a foreign patent office in a counterpart application, and
- (2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

- (1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim;
- or
- (2) It refutes, or is inconsistent with, a position the applicant takes in:
    - (i) Opposing an argument of unpatentability relied on by the Office, or
    - (ii) Asserting an argument of patentability.

A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability.

- (c) Individuals associated with the filing or prosecution of a patent application within the meaning of this section are:
  - (1) Each inventor named in the application;
  - (2) Each attorney or agent who prepares or prosecutes the application; and
  - (3) Every other person who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application.

(d) Individuals other than the attorney, agent or inventor may comply with this section by disclosing information to the attorney, agent, or inventor.

(e) In any continuation-in-part application, the duty under this section includes the duty to disclose to the Office all information known to the person to be material to patentability, as defined in paragraph (b) of this section, which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby appoint the following attorney(s) and/or patent agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith:

Albrecht, John W.	Reg. No. 40,481	Leonard, Christopher J.	Reg. No. 41,940
Ali, M. Jeffer	Reg. No. 46,359	Liepa, Mara E.	Reg. No. 40,066
Anderson, Gregg I.	Reg. No. 28,828	Lindquist, Timothy A.	Reg. No. 40,701
Batzli, Brian H.	Reg. No. 32,960	Lycke, Lawrence E.	Reg. No. 38,540
Beard, John L.	Reg. No. 27,612	Mayfield, Denise L.	Reg. No. 33,732
Berns, John M.	Reg. No. 43,496	McDonald, Daniel W.	Reg. No. 32,044
Black, Bruce E.	Reg. No. 41,622	McIntyre, Jr., William F.	Reg. No. 44,921
Branch, John W.	Reg. No. 41,633	Mitchem, M. Todd	Reg. No. 40,731
Bremer, Dennis C.	Reg. No. 40,528	Mueller, Douglas P.	Reg. No. 30,300
Bruss, Steven C.	Reg. No. 34,130	Nichols, A. Shane	Reg. No. 43,836
Byrne, Linda M.	Reg. No. 32,404	Parsons, Nancy J.	Reg. No. 40,364
Campbell, Keith	Reg. No. 46,597	Pauly, Daniel M.	Reg. No. 40,123
Carlson, Alan G.	Reg. No. 25,959	Phillips, Bryan K.	Reg. No. 46,990
Caspers, Philip P.	Reg. No. 33,227	Phillips, John B.	Reg. No. 37,206
Chiapetta, James R.	Reg. No. 39,634	Prendergast, Paul	Reg. No. 46,068
Clifford, John A.	Reg. No. 30,247	Pytel, Melissa J.	Reg. No. 41,512
Coldren, Richard J.	Reg. No. 44,084	Qualey, Terry	Reg. No. 25,148
Daignault, Ronald A.	Reg. No. 25,968	Reich, John C.	Reg. No. 37,703
Daley, Dennis R.	Reg. No. 34,994	Reiland, Earl D.	Reg. No. 25,767
Dalglish, Leslie E.	Reg. No. 40,579	Roberts, Fred	Reg. No. 34,707
Daulton, Julie R.	Reg. No. 36,414	Samuels, Lisa A.	Reg. No. 43,080
DeVries Smith, Katherine M.	Reg. No. 42,157	Schmaltz, David G.	Reg. No. 39,828
DiPietro, Mark J.	Reg. No. 28,707	Schuman, Mark D.	Reg. No. 31,197
Edell, Robert T.	Reg. No. 20,187	Schumann, Michael D.	Reg. No. 30,422
Epp Ryan, Sandra	Reg. No. 39,667	Scull, Timothy B.	Reg. No. 42,137
Glance, Robert J.	Reg. No. 40,620	Sebal, Gregory A.	Reg. No. 33,280
Goggin, Matthew J.	Reg. No. 44,125	Skoog, Mark T.	Reg. No. 40,178
Golla, Charles E.	Reg. No. 26,896	Spellman, Steven J.	Reg. No. 45,124
Gorman, Alan G.	Reg. No. 38,472	Stoll-DeBell, Kirstin L.	Reg. No. 43,164
Gould, John D.	Reg. No. 18,223	Sumner, John P.	Reg. No. 29,114
Gregson, Richard	Reg. No. 41,804	Swenson, Erik G.	Reg. No. 45,147
Gresens, John J.	Reg. No. 33,112	Tellekson, David K.	Reg. No. 32,314
Hamer, Samuel A.	Reg. No. 46,754	Trembath, Jon R.	Reg. No. 38,344
Hamre, Curtis B.	Reg. No. 29,165	Tuchman, Ido	Reg. No. 45,924
Harrison, Kevin C.	Reg. No. 46,759	Tunheim, Marcia A.	Reg. No. 42,189
Hertzberg, Brett A.	Reg. No. 42,660	Underhill, Albert L.	Reg. No. 27,403
Hillson, Randall A.	Reg. No. 31,838	Vandenburgh, J. Derek	Reg. No. 32,179
Holzer, Jr., Richard J.	Reg. No. 42,668	Wahl, John R.	Reg. No. 33,044
Johnston, Scott W.	Reg. No. 39,721	Weaver, Karrie G.	Reg. No. 43,245
Kadievitch, Natalie D.	Reg. No. 34,196	Welter, Paul A.	Reg. No. 20,890
Karjeker, Shaukat	Reg. No. 34,049	Whipps, Brian	Reg. No. 43,261
Kettelberger, Denise	Reg. No. 33,924	Whitaker, John E.	Reg. No. 42,222
Keys, Jeramie J.	Reg. No. 42,724	Wickhem, J. Scot	Reg. No. 41,376
Knearl, Homer L.	Reg. No. 21,197	Williams, Douglas J.	Reg. No. 27,054
Kowalchuk, Alan W.	Reg. No. 31,535	Withers, James D.	Reg. No. 40,376
Kowalchuk, Katherine M.	Reg. No. 36,848	Witt, Jonelle	Reg. No. 41,980
Lacy, Paul E.	Reg. No. 38,946	Wu, Tong	Reg. No. 43,361
Larson, James A.	Reg. No. 40,443	Xu, Min S.	Reg. No. 39,536
Leon, Andrew J.	Reg. No. 46,869	Zeuli, Anthony R.	Reg. No. 45,255

I hereby authorize them to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/ organization who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct Merchant & Gould P.C. to the contrary.

I understand that the execution of this document, and the grant of a power of attorney, does not in itself establish an attorney-client relationship between the undersigned and the law firm Merchant & Gould P.C., or any of its attorneys.

[illegible]

**23552**  
PATENT TRADEMARK OFFICE

 $1-OC$ 2-003-04

\*\*\*\*\*  
\*\*\* RX REPORT \*\*\*  
\*\*\*\*\*

RECEPTION OK

TX/RX NO	8706	
CONNECTION TEL		228 9446
SUBADDRESS		
CONNECTION ID	KLARQUIST SPARKM	
ST. TIME	01/08 17:15	
USAGE T	02'49	
PGS.	10	
RESULT	OK	

SEQUENCE LISTING

<110> The Government of the United States of America as

<120> VARIANTS OF HUMANIZED ANTI-CARCINOMA MONOCLONAL  
ANTIBODY CC49

<130> 11613.32WO01

<140> PCT/US99/25552

<141> 1999-10-29

<150> 60/106,534

<151> 1998-10-31

<150> 60/106,757

<151> 1998-11-02

<160> 44

<170> PatentIn Ver. 2.0

<210> 1

<211> 17

<212> PRT

<213> Mus musculus

<400> 1

Lys	Ser	Ser	Gln	Ser	Leu	Leu	Tyr	Ser	Gly	Asn	Gln	Lys	Asn	Tyr	Leu
1				5					10					15	

Ala

<210> 2

<211> 7

<212> PRT

<213> Mus musculus

<400> 2

Trp	Ala	Ser	Ala	Arg	Glu	Ser
1				5		

<210> 3

<211> 9

<212> PRT

<213> Mus musculus

<400> 3

Gln Gln Tyr Tyr Ser Tyr Pro Leu Thr  
1 5

<210> 4

<211> 5

<212> PRT

<213> Mus musculus

<400> 4

Asp His Ala Ile His  
1 5

<210> 5

<211> 17

<212> PRT

<213> Mus musculus

<400> 5

Tyr Phe Ser Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu Arg Phe Lys  
1 5 10 15

Gly

<210> 6

<211> 6

<212> PRT

<213> Mus musculus

<400> 6

Ser Leu Asn Met Ala Tyr  
1 5

<210> 7

<211> 17

<212> PRT

<213> Homo sapiens

<400> 7

Lys Ser Ser Gln Ser Val Leu Tyr Ser Ser Asn Ser Lys Asn Tyr Leu  
1 5 10 15



<210> 12  
 <211> 12  
 <212> PRT  
 <213> Homo sapiens

<400> 12  
 Gly Gly Tyr Tyr Gly Ser Gly Ser Gly Ser Asn Tyr  
 1 5 10

<210> 13  
 <211> 113  
 <212> PRT  
 <213> Mus musculus

<400> 13  
 Asp Ile Val Met Ser Gln Ser Pro Asp Ser Leu Ala Val Ser Leu Gly  
 1 5 10 15  
 Glu Arg Val Thr Leu Asn Cys Lys Ser Ser Gly Ser Leu Leu Tyr Ser  
 20 25 30  
 Gly Asn Gln Lys Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln  
 35 40 45  
 Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser Ala Arg Glu Ser Gly Val  
 50 55 60  
 Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr  
 65 70 75 80  
 Ile Ser Ser Val Gln Ala Glu Asp Val Ala Val Tyr Tyr Cys Gln Gln  
 85 90 95  
 Tyr Tyr Ser Tyr Pro Leu Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu  
 100 105 110

Lys

<210> 14  
 <211> 115  
 <212> PRT  
 <213> Mus musculus

<400> 14  
 Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Val Lys Pro Gly Ala



1 5 10 15  
 Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp His  
 20 25 30  
 Ala Ile His Trp Val Lys Gln Asn Pro Gly Gln Arg Leu Glu Trp Ile  
 35 40 45  
 Gly Tyr Phe Ser Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu Arg Phe  
 50 55 60  
 Lys Gly Lys Ala Thr Leu Thr Ala Asp Thr Ser Ala Ser Thr Ala Tyr  
 65 70 75 80  
 Val Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Phe Cys  
 85 90 95  
 Thr Arg Ser Leu Asn Met Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr  
 100 105 110  
 Val Ser Ser  
 115

<210> 15  
 <211> 124  
 <212> DNA  
 <213> Mus musculus

<400> 15  
 ctaagcttcc accatggagt ggtcctgggt ctccctcttc ctccctgctgc tgtgggtgag 60  
 gggaccctgt gccggtccct gagtcgcggc ctgacgtggt cgacctggac cctcacgtga 120  
 cgtg 124

<210> 16  
 <211> 123  
 <212> DNA  
 <213> Mus musculus

<400> 16  
 ggagagaaat atccaatcca ctccaggcgc tgtccaggat tctgtttcgc acttctaaag 60  
 gacgttccgt tcgccgatgt ggaagtgaga gatatcgctt ttagtcttcc cagggacacg 120  
 gcc 123

<210> 17  
 <211> 126  
 <212> DNA  
 <213> Mus musculus



<212> DNA

<213> Mus musculus

<400> 22

```
agcccataat atcgataggg gagtgtgaagc cgcgaccgtg gttcgacctt gactttgccc 60
ggcgtgctga caataataga ctgccacgtc ttctgcctgc acgctgctga ttgtcagagt 120
gaagtc                                           126
```

<210> 23

<211> 19

<212> DNA

<213> Artificial

<400> 23

ctaagcttcc accatggag 19

<210> 24

<211> 19

<212> DNA

<213> Artificial

<400> 24

atgggcccgt agtttggcg 19

<210> 25

<211> 20

<212> DNA

<213> Artificial

<400> 25

gcaagcttcc accatggata 20

<210> 26

<211> 20

<212> DNA

<213> Artificial

<400> 26

agccgcggcc cgtttcagtt 20

<210> 27

<211> 42

<212> DNA

<213> Artificial

<400> 27

gccagcgccg aagctgaggg gatagctata atactgctga ca 42

<210> 28  
 <211> 45  
 <212> DNA  
 <213> Artificial  
  
 <400> 28  
 ggtgccagcg ccgaagctga ggggggtgct ataatactgc tgaca 45  
  
 <210> 29  
 <211> 42  
 <212> DNA  
 <213> Artificial  
  
 <400> 29  
 gccacggccg aatgtgtagg gatagctata atactgctga ca 42  
  
 <210> 30  
 <211> 39  
 <212> DNA  
 <213> Artificial  
  
 <400> 30  
 gccgaatgtg aggggggtgc tataatactg ctgacaata 39  
  
 <210> 31  
 <211> 37  
 <212> DNA  
 <213> Artificial  
  
 <400> 31  
 gtttcaccca gtgcattgca taatcagtga aggtgta 37  
  
 <210> 32  
 <211> 56  
 <212> DNA  
 <213> Artificial  
  
 <400> 32  
 gtggccttgc cctggaactt ctgtgagtac ttaaaatcat cgtttccggg agagaa 56  
  
 <210> 33  
 <211> 23  
 <212> PRT  
 <213> Homo sapiens  
  
 <400> 33  
 Asp Ile Val Met Thr Gln Ser Pro Asp Ser Leu Ala Val Ser Leu Gly  
 1 5 10 15



1 5 10 15  
 Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr  
 20 25 30

<210> 38  
 <211> 14  
 <212> PRT  
 <213> Homo sapiens

<400> 38  
 Trp Val Arg Gln Ala Pro Gly Gln Arg Leu Glu Trp Met Gly  
 1 5 10

<210> 39  
 <211> 32  
 <212> PRT  
 <213> Homo sapiens

<400> 39  
 Arg Val Thr Ile Thr Arg Asp Thr Ser Ala Ser Thr Ala Tyr Met Glu  
 1 5 10 15

Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg  
 20 25 30

<210> 40  
 <211> 11  
 <212> PRT  
 <213> Homo sapiens

<400> 40  
 Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser  
 1 5 10

<210> 41  
 <211> 423  
 <212> DNA  
 <213> Mus musculus

<400> 41

gcaagcttcc accatggata gccaggccca ggtgctcatg ctctgctgc tgtgggtgag 60  
 cggcacatgc ggcgacatcg tgatgagcca gtctccagac tccctggccg tgtcccaggg 120  
 cgagaggggtg actctgaatt gcaagtcagc cagtcctgc tctatagcgg aaatcagaag 180  
 aactatctcg cctggtatca gcagaaacca ggcagagcc cttaaactgct gatttactgg 240  
 gcatccgcta ggaatccgg cgtgcctgat cgcttcagcg gcagcggatc tgggacagac 300  
 ttactctga caatcagcag cgtgcaggca gaagacgtgg cagtctatta ttgtcagcag 360  
 tattatagct atcccctcac attcggcgct ggcaccaagc tggaactgaa acggggcccg 420  
 gct 423

<210> 42

<211> 424

<212> DNA

<213> Mus musculus

<400> 42

agccgcgcc cggttcagtt ccagcttggg gccagcgccg aatgtgaggg gatagctata 60  
 atactgctga caataataga ctgccacgtc ttctgctgc acgctgctga ttgtcagagt 120  
 gaagtctgtc ccagatccgc tgcgctgaa gcgatcaggc acgcccggatt ccctagcggg 180  
 tgcccagtaa atcagcagtt tagggctctg ccctggtttc tgctgatacc aggcgagata 240  
 gttcttctga ttccgctat agagcagggg ctggctggac ttgcaattca gagtcaccct 300  
 ctgcgccagg gacacggcca gggagtctgg agactggctc atcacgatgt cgcgcgatgt 360  
 gccgctcacc cacagcagca ggagcatgag cacctgggccc tggctatcca tgggtggaagc 420  
 ttgc 424

<210> 43

<211> 434

<212> DNA

<213> Mus musculus

<400> 43

ctaagcttcc accatggagt ggtcctgggt ctctctcttc ctctgctgc tgtgggtgag 60  
 agtgcactcc caggtccagc tgggtcagtc cggcgctgag tccctggccg tgtcccaggg 120  
 cgtgaagatt tctgcaagg caagcggcta cacttctact ctctatagcg gaaatcagaa 180  
 gaaacagaat cctggacagc gcctggagtg gattggatat ttctctcccg gaaacgatga 240  
 ttttaagtac aatgagaggt tcaagggcaa ggccacactg actgcagaca catctgccag 300  
 cactgcctac gtggagctct ccagcctgag atccgaggat actgcagtgt acttctgcac 360  
 aagatccctg aatatggcct actggggaca gggaaccctg gtcaccgtct ccagcgccaa 420  
 aactacgggc ccat 434

<210> 44

<211> 434

<212> DNA

<213> Mus musculus

<400> 44

atgggcccgt agttttggcg ctggagacgg tgaccagggg tccctgtccc cagtaggcca 60  
 tattcagggg tcttgtgcag aagtacactg cagtatcttc ggatctcagg ctggagagct 120  
 ccacgtaggc agtgctggca gatgtgtctg cagtcagtgt ggccttgccc ttgaacctct 180

cattgtactt aaaatcatcg ttccgggag agaaatatcc aatccactcc aggcgctgtc 240  
caggattctg tttcttctga ttccgctat agagagtga ggtgtagccg ctgaccttgc 300  
aggaaatctt cacgcccagg gacacggcca gggactcagc gccggactgc accagctgga 360  
cctgggagtg cactctcacc cacagcagca ggaggaagag gaagaccag gaccactcca 420  
tggtggaagc ttag 434